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## Smallpox: ignorance is never bliss

IGNORANCE confronts ignorance in the case of the release of the smallpox virus, *Variola major* Abid, from a Birmingham University laboratory last August. Clive Jenkins, leader of the Association of Scientific, Technical and Managerial Staffs (ASTMS), a trades union taking an increasing interest in laboratory safety in the UK, 'published' the confidential Shooter Report on the Birmingham affair last week. This has removed our ignorance of the events which led up to the smallpox release, as we describe in the next few pages; but it has not removed our ignorance of the smallpox virus, whose relationships among its many strains and to the various animal poxes—from which it might re-emerge—remains obscure. Professor Henry Bedson was working on the protein characterisation of the various pox viruses, under pressure from the forthcoming closure of his laboratory, when the escape occurred. The World Health Organisation (WHO) team, whose work has led to the virtual eradication of *Variola major* as a cause of disease, had earlier in the year described his work as "fascinating", but within a few weeks Professor Bedson had committed suicide.

Professor Bedson, the Shooter Report implies, had been guilty of his own kind of ignorance: wilfully or otherwise he had ignored elementary safety precautions in the handling of the virus. According to one member of the Dangerous Pathogens Advisory Group (DPAG), which licensed Bedson's laboratory as adequate in 1976, the equipment Bedson was using was unsafe "except for the mildest pathogens". Interestingly enough, the first this DPAG member had seen of the apparatus was on television news last week.

None of these various kinds of ignorance is tolerable. First we must know more of the smallpox virus and its relatives. Second, those working with pathogenic—or potentially pathogenic—organisms must be prepared to educate themselves about the mechanisms by which those organisms can escape. (This involves as much physics as biology). And third, after DPAG is disbanded (it must be, if only to restore public confidence), its successor must be provided with sufficient critical investigative power that its members do not have to rely on the television for their information.

Most of what needs to be done follows from these three requirements. First, Professor Dumbell, whose laboratory at St Mary's Hospital is the only one to retain smallpox virus in the UK, must be given the means to continue his work (the characterisation of pox viruses by their DNA content). It is not reasonable that he should be hounded into a two-year hiatus in his work because of the laxity of another laboratory. The World Health Organisation, which suffered no strictures in Shooter for its inspection

procedures, has designated Dumbell's laboratory as one of the only four in the world that can continue to work on smallpox. He should be able to continue. But perhaps he should be prepared to take up the offer, reported below, of the director of the special pathogens unit at Porton Down, Dr David Simpson, to store his smallpox samples at Porton (whose containment facilities are second to none) and provide him with laboratory space. This would imply that Professor Dumbell's work could continue soon, but in collaboration with Porton staff; and that his animal pox samples—which are not so virulent as *Variola major*—would remain at St Mary's. Such an arrangement might prove an effective compromise, if logic rather than emotion prevails.

Second, the need for improving safety measures and safety education at other laboratories holding category A pathogens (and for that matter the milder category B pathogens too) leads to a number of consequences: that money should be made readily available for increasing laboratory safety (it has not been easily available in the past); that safety officers be provided with sufficient status that they can confront heads of departments on equal terms; and that (we shall argue) a body be set up to provide education and research into biosafety.

That safety money has not come easily in the past is illustrated by the comment, only last week, of Professor Arie Zuckerman of the London School of Hygiene and Tropical Medicine that he could not afford the six white coats per staff member recommended in the Godber report (the result of an inquiry which followed a smallpox outbreak at the school in 1973). Presumably, in the present political climate, money should present no problem. But it must be provided in such a way that there is no conflict of interest over its spending. If it were provided, for example, by the Medical Research Council (MRC), which funds biomedical research, there would be a regular conflict over how much to spend on research and how much on safety. Again, if that money were provided as a block grant to a head of department, there might be an accounting difficulty in determining whether the money had finally been spent on safety or on some other matter. The best solution would seem to be money provided either by the ministry concerned (the Department of Education and Science for universities and the Department of Health and Social Security for hospitals) or better directly by the Health and Safety Executive (HSE) to the safety officer of each laboratory. Then the conflicts of interest in the giving and the spending of money would be minimised.

Status for the safety officer is a thornier problem. It is not merely a matter of equal salaries, but one of prestige, age, and personality. There must be a mutual respect for



their individual concerns between the head of department and the safety officer, the one for the academic advancement of the department, the other for the human welfare of its members. Probably the most experienced and knowledgeable biosafety officer in this country, Dr Mark Darlow (who was head of the safety section at Porton from 1951 to 1976), believes that the job involves being "a kind of uncle". He is a doctor of medicine, and has something of the manner of a general practitioner—firm yet sympathetic—and believes it vital that a safety officer gets to know the families of the people in his care, and their doctors. He has all the assurance of the medical profession, and one can be quite sure he had the confidence to confront the director of Porton and its scientists when it was necessary. And he was successful, despite the awesome panoply of bugs (from smallpox to plague) that Porton carried. There were very few incidents, and those only due to inevitable human frailty. It is dangerous to argue from one case; but there *are* very few; and it seems reasonable to suggest that a safety officer should always be a doctor. This gives him or her the status and confidence of the profession, and ensures the right motivation; safety is not a business for failed academics.

This leads to the next matter—the need for education and research in biosafety. Darlow's confidence rests not only on his professional sangfroid but also on experiment. In very early days at Porton, for example, he discovered the hardness of the smallpox virus. He found that it can survive in dust in the corners of rooms, in pockets, even in dust on the top of a fluorescent light. He did experiments with high speed cinephotography on pipetting, and showed that a cloud of tiny droplets floats into the air with every drop released. He had the confidence of facts, and could convince the scientists in his care with evidence. No scientist is going to accept arbitrary controls; there must be adequate reason.

Not only is there now very little research being done (whereas as great deal remains to be learned), but what is known is not widely disseminated. As Dr Darlow wrote prophetically in 1969 "The literature on the subject is in fact very extensive, but many of the publications have appeared in unfamiliar journals and have consequently been overlooked; it is to be regretted that the spilling of ink is so inaudible and that positive action is only stimulated by the resounding spilling of pathogens." (*Methods in Microbiology* vol 1 ch 6; 1969.)

Biosafety is peripheral to the interests of an ambitious academic, and he or she is not going to spend as long reading the safety literature as reading the latest development in his or her field. That much is inevitable. Neither is such a person going to spend much time researching hazards, the reward for which is a human one quite different from the pursuit of academic success. It is therefore necessary to set up a new institution, with its own career structure and motivations, with the objective both of disseminating existing scientific knowledge on biosafety, and, most important for maintaining the liveliness of the body, with the objective of pursuing research into biosafety questions. There are many of these, and not only concerning work with known pathogens; there is a great deal yet to be learned about the safety of work with recombinant DNA, for example.

Further, since one product of such a unit would certainly be ideas for new devices to increase containment and ease research, and since such devices will be in demand, the unit could even generate some of its own income through patents and licensing. If Britain moved fast enough, such a body could become a world leader in the field whose prestige would make it easier to attract the right kind of talents to it: talents in education, medicine, biology, physics, and commerce. The Secretary of State for Education

and Science, Mrs Shirley Williams, who recently indicated her willingness to invest large sums of money in research into the hazards of genetic engineering, would be well advised to consider setting up such an institution. It would be an innovation. None yet exists quite like it; neither the National Radiological Protection Board, nor the laboratories of the Health and Safety Executive, nor the research units of the MRC, have such conjoined functions; but the functions must be conjoined as each activity would stimulate the others. Alone, each is a stranded whale.

After continuing research into smallpox; after removing ignorance of biosafety; the third matter we raised was the question of keeping DPAG's successor properly informed. Of course that in itself is not enough: DPAG proved itself unwilling to act. But a dose of 'public interest' and union representation, as on the Genetic Manipulation Advisory Group, should soon cure that. DPAG already had teeth, under the Health and Safety at Work Act; its advice had to be accepted or a laboratory would have to close. Its successor needs no more than that—except information.

DPAG was not well informed. It has emerged now that there is no complete and guaranteed list of the laboratories holding category A pathogens, let alone category B. The Godber working party, which defined the categories, sent out a large number of questionnaires concerning pathogens held to as many laboratories as they could think of. Of 4,016 laboratories questioned, 2,152 did not even reply. Perhaps they held no pathogens; but there is no guarantee of that. Further, DPAG has had only one full time inspector, a retired member of the Public Health Services Laboratory, plus two occasional assistants, to inspect the 115 laboratories holding category A pathogens that *were* unearthed by Godber. At Birmingham, according to Shooter, the DPAG inspectors failed even to consult a checklist of items recommended by the Godber report.

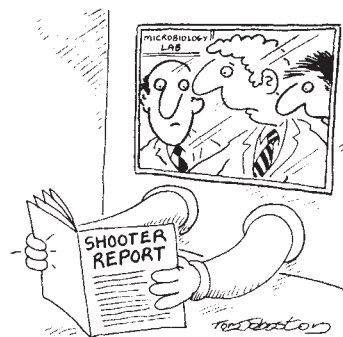
The Health and Safety Executive has a special unit of three microbiologists, but that is less than a year old and is now occupied full time until April inspecting 11 laboratories holding category A human pathogens. (Incidentally DPAG has reduced the list of 12 category A human pathogens defined by Godber to 5, while adding 4 new ones.) This same HSE team also has the duty of inspecting laboratories for the Genetic Manipulation Advisory Group. It is clearly overstretched, and must be expanded by a large factor. But there is another problem: where are people of sufficient quality and knowledge to be found? The HSE function must not be one of rubber-stamping, of accepting the assurances of the 'expert' at the laboratory who rarely turns to the biosafety literature. There may be no more than half a dozen people in the country who really understand the field. These people must be brought together as a matter of urgency, first to assist in training new inspectors for the HSE and second to advise on the setting up of the biosafety research and education unit we recommend. Ultimately that unit can become the source of new inspectors and of biosafety officers.

Finally, there is the question of the need for another public inquiry, as demanded by Mr Clive Jenkins. Our view is that would only confuse a situation where a laboratory is already littered with the recommendations of well-meaning committees. The Godber working party was set up five years ago to clarify the situation and that it did. The failure has been to implement its recommendations properly, which is a failure of government. DPAG should be reconstituted along the lines of GMAG. The HSE microbiological inspectorate should be significantly strengthened. A biosafety research and education unit should be set up. And a way should be found to enable Professor Dumbell to continue his smallpox work rapidly, probably at Porton. These are the ways forward from Birmingham. □



On 24 August last year Professor Henry Bedson of the Department of Medical Microbiology at Birmingham University analysed samples from Mrs Janet Parker, a university photographer, and confirmed that she had smallpox. The source of her infection was almost certainly Bedson's smallpox laboratory. Within a few weeks Professor Bedson had committed suicide and Mrs Parker had died. The affair is *sub judice* under the pending court case against Birmingham University under the Health and

Safety at Work Act, but despite that Clive Jenkins, leader of the Association of Scientific, Technical and Managerial Staffs last week published his copy of the Shooter report—the confidential government report of the committee of inquiry into the incident. Shooter's indictment is a strong one, and Mr Jenkins believed it in "the over-riding public interest" that he publish it. Here Robin McKie of the *Times Higher Education Supplement*, Mary Lindley, Judy Redfearn and Robert Walgate report.



## UK smallpox research could continue at Porton

The unofficial publication of the Shooter report by union leader Clive Jenkins last week is likely to produce swift results in Britain. The recommendation that the nation's only remaining smallpox research unit, at St Mary's Hospital in London, should be moved to an isolated area in the country has already been backed by the local Member of Parliament, Mr Arthur Latham. And the head of the unit, Professor Keith Dumbell, admitted that it would now be impossible for smallpox research to be carried out at St Mary's.

However, later in the week Dr David Simpson, recently appointed director of the special pathogens unit at the Medical Research Establishment, Porton, told *Nature* that he would be willing to receive Professor Dumbell's live smallpox virus at Porton. He could store it there, said Dr Simpson, but he did not want Porton to become "just rent-a-space". Any researcher using Porton in this way would be expected to use and collaborate with Porton staff in research on the material so stored.

Professor Dumbell, on the other hand, believes the scientific case for moving is arguable. "We have in our laboratory all the precautions that we

have been able to think up. The chances of a virus escaping are very much smaller than those risks to which we expose ourselves everyday", Professor Dumbell added.

His laboratory was a purpose-built unit designed to cope with smallpox research and took two years to set up. The safeguards—in contrast to the inadequate measures disclosed at Birmingham—include a sealed anteroom; effective laboratory air filtration; anti-septic disposable clothing; a shower room; and regular vaccinations. The move to the country would cost two years' effort that could otherwise be used for smallpox research, he stated.

If the smallpox laboratory at St Mary's had to close it would be for political reasons, added the school's dean, Dr Harold Edwards. The move to the country would only serve to isolate the smallpox researchers from colleagues involved in similar areas of work. And Dr Edwards warned that the controversy surrounding the Birmingham smallpox outbreak could lead to growing demands for tighter controls which would diminish the quantity of scientific research in Britain. "The trouble is that increases in controls are not followed by the necessary increases in public funds.

Something else has to be taken out of research funds to achieve new safety standards".

Professor Dumbell, who supplied Professor Bedson at Birmingham with 22 virus strains including the one which killed Mrs Parker, admitted there had been a misdemeanour involved when they did not inform the Dangerous Pathogens Advisory Group (DPAG) of the transfer. He thought both of them had assumed the other had notified the group.

Professor Bedson and Professor Dumbell were working on parallel lines of research. At Birmingham, Professor Bedson was using the protein coats of viruses as the basis of an identification process, while Professor Dumbell was attempting a similar technique using the DNA of viruses.

Asked if Professor Bedson could have carried on his work at St Mary's instead of rushing to complete it at Birmingham, Professor Dumbell said that when the World Health Organisation indicated it was to close down all smallpox research units in Britain, apart from the one at St Mary's, he had been asked by the researchers if he would provide space for them to continue their work. "I answered that I was prepared to do this". □



Professor Keith Dumbell (left) and the sort of facilities at Porton (right) with which he could continue smallpox research.

## How the virus escaped

THE Birmingham smallpox laboratory which was the source of the infection which killed Mrs Janet Parker, is a tiny, eight-foot square room in the east wing of Birmingham University's medical school. It is surrounded by a larger room in which experiments on animal pox viruses were performed. Both rooms contain equipment used in normal microbiological research, including safety cabinets with air filters, centrifuges and autoclaves for sterilising gowns and equipment.

The Shooter committee firstly found there was no evidence that Mrs Parker had ever been in the pox virus labora-



tory suite and they concluded that the virus must therefore have escaped from the smallpox room. The group then discovered that not all smallpox work had been carried out inside the room's safety cabinet and some work with an aspirator to suck off fluid from cell cultures had been undertaken on an open bench.

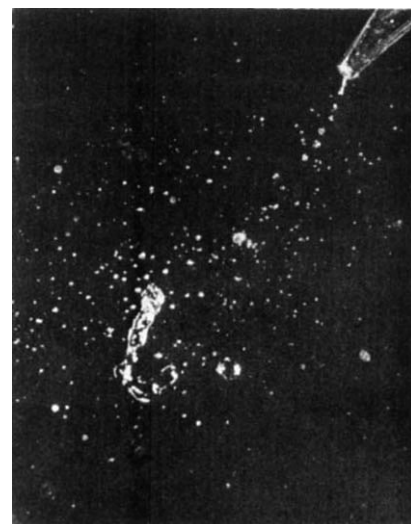
The principal means of transmission of smallpox viruses is probably by aerosol, in tiny droplets of fluid that form when a pipette is used to deliver drops onto a gel or whenever there is splashing of fluid containing the virus. According to Dr Mark Darlow, head of the safety department at the Porton germ warfare establishment for 25 years, the virus is also very stable; so that once airborne it is a potential killer. An efficient air withdrawal and filtration system over any experiment is therefore essential. But many academics still believe that "what was good enough for Pasteur is good enough for me", said Dr Darlow last week.

According to the Shooter report "the opening and closing of the smallpox room door and the passage in and out by whoever was conducting work on the virus would have created the opportunity for any airborne virus to escape into the animal pox room".

The consequent danger was made all the worse because gowns worn in the smallpox room were not removed on leaving.

Even more seriously, the service ducts in the animal pox room and the smallpox room both had gaps which could allow the leakage of viruses. In particular, the telephone room above, which was connected by the duct to the animal pox room, was used frequently by Mrs Parker, when she was telephoning suppliers to order photographic materials. "A check of the orders placed by Mrs Parker during this period reveals that on 25 July she placed an unusually large number of orders. The relevant strain of smallpox virus, Abid, was being handled in the smallpox room on July 24 and 25", the report states.

The committee, although not certain by what route Mrs Parker was infected, concluded that escape via the service duct in the animal pox room was therefore the most probable route of escape of the virus. However, the virus could have reached the corridor outside the laboratory suite and Mrs Parker could have been infected when visiting the inquiry office or the dark-room at the end of the corridor. This was a less likely route, though, and was



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*An aerosol resulting from a bubble burst at a pipette tip when the last drop was expelled. This explains how smallpox virus might have escaped into the air.*

used by many other persons.

And although they could not be certain about the exact route, the Shooter committee was certain that a combination of poor laboratory procedures, amounting to a major breach in containment policy, was responsible for the outbreak. □

## All safety nets failed, says Shooter

The report of the Shooter inquiry into the causes of the smallpox outbreak in Birmingham last year finds fault with the three major organisations—the Dangerous Pathogens Advisory Group (DPAG), the World Health Organisation (WHO) and Birmingham University—which were concerned in some way with either running or monitoring the smallpox laboratory at the Department of Medical Microbiology at Birmingham University.

### DPAG

The Dangerous Pathogens Advisory Group (DPAG) exists precisely to prevent the sort of event which occurred at Birmingham. It was created in 1975 in the wake of a previous outbreak of smallpox from a laboratory in London in 1973. Shortly after it came into being it began to formulate a code of practice for work with category A pathogens—those recognised as the most dangerous—and to inspect all laboratories known to hold them.

In February 1976, DPAG's inspector visited the Birmingham laboratory. During the time he was there, says the Shooter report, no work on smallpox was being done. He tested the airflow through the safety cabinet and then spent most of his visit talking to Professor Bedson about smallpox work.

The Shooter report criticises the DPAG inspector for not finding out enough about the "range and extent of the work being done". He did not, says the report, "ask about work with tissue cultures"; neither did he ask about the methods of harvesting virus. "These points seem to us to be of considerable importance", the report goes on, "since one of the unsatisfactory features . . . was the necessity to pass in and out of the smallpox room during the course of work with smallpox to place cultures in the incubators and to use the low speed centrifuge".

Despite the fact that the Birmingham lab lacked some of the facilities then recommended for use with category A pathogens, namely an air lock, shower, changing facilities and double autoclave, the DPAG inspector recommended to DPAG that the laboratory be approved. He based his judgement on Professor Bedson's reputation as an "experienced and safety-conscious virologist" and the fact that the few named people working on smallpox always did so under Bedson's supervision. There was also a "highly efficient vaccination programme" in force.

When DPAG came to discuss the inspector's report (it meets twice yearly) it felt that it could use its discretionary powers—granted to it when

it was created—to recommend approval of the laboratory to the DHSS despite the shortcomings. It added, however, that "fresh clearance should be sought in the event of significant changes in staff, facilities or work programme".

The committee of inquiry felt that the inspector's report did not give DPAG sufficient information on which to base a recommendation. It criticises DPAG for not insisting on an inspection report that compared the facilities and procedures with those laid out in its own safety code and recommends that in future DPAG inspectors should compare laboratories' facilities against a detailed check list. It also criticises the way in which DPAG exercised its discretionary powers and recommends that in future "discretion should be exercised by DPAG only if alternative arrangements are in force in a category A laboratory which are able to achieve a degree of safety equivalent to that specified in the safety code."

Since the inspection in 1976, says the report, changes had taken place in the smallpox laboratory. Professor Bedson had ceased to do experiments because of other commitments and most of the work had been taken over by a PhD student. This had not been explained to the DPAG inspector. In addition



*St Mary's Hospital, London which houses the UK's only smallpox laboratory.*

the volume of work increased. Twenty two additional strains of variola virus were delivered to the laboratory in early 1978.

DPAG, says the report, was notified of none of these changes. The report therefore recommends that as well as notifying DHSS of any changes, category A laboratories should be reviewed annually. It also suggests that notification of intention to hold category A pathogens should be compulsory and that DPAG should start inspecting all other category A laboratories immediately. Finally, it says, the need to work with variola virus in the UK should be reviewed. If it is decided that work should go on, then, the report recommends, the one remaining laboratory in the UK holding the virus, at St. Mary's Hospital, London, should be moved into the country.

#### **Birmingham University**

Birmingham University and the Department of Medical Microbiology both had safety policies at the time of the smallpox outbreak which should have had some influence on the way research on smallpox was conducted. The committee of inquiry found, however, that there was no "effective system of determining whether both . . . safety policies were being regularly implemented".

The responsibility of putting the safety codes into practice rested with Professor Bedson as head of department. Responsibility for formulating the safety code specific to microbiological research, however, rested with the committee for the control of pathogenic organisms and infectious

materials (CCPOIM), a subcommittee of the university's central safety committee, whose members came solely from the academic staff.

Since the inspection of all departments working with microorganisms by CCPOIM in 1975 there had been no further inspections, says the report. It therefore recommends that "expert safety inspections" should be carried out regularly. It also suggests that a head of department should not be a "member of the team that inspects his own department" (in 1975, Professor Bedson's had been one of the inspecting team), and that the membership of the CCPOIM be extended to include members of the non-academic university staff.

The committee of inquiry also found the experience of the staff working in the smallpox laboratory lacking. Apart from a technician who had worked with smallpox for 11 years and had been trained by Professor Bedson, the other people working on smallpox were a former PhD student, who joined the laboratory in 1974, and a trainee technician who had been working at the lab for about a year. "As far as we know", says the report, the former PhD student "was never formally trained in the special precautions required for work with smallpox viruses". The trainee technician, who joined the lab straight after leaving school, had apparently started working with smallpox virus "only nine months after she had joined the laboratory".

The report says that this state of affairs was inadequate. It recommends that the university review its policy for instructing staff in laboratory techniques and safety precautions and that it ensures that "the staff are not permitted to carry out such work without appropriate . . . supervision".

#### **WHO**

The WHO had been corresponding with Professor Bedson about safety without the knowledge of either the Birmingham University authorities or the DHSS.

"It is anomalous", says the report, "that though WHO had decided the work could not be supported after the end of 1978 this was not communicated to DHSS or the university". It recommends that in future "institutions should ensure that all dealings with outside bodies concerning work with safety implications in their departments are monitored by the central administration. . . ." Its recommendation to the WHO is that it should "maintain a closer liaison with the responsible government authority regarding its dealings with category A pathogen laboratories and in particular with regard to the safety of those laboratories". □

## **WHO had serious doubts on safety in smallpox lab**

WHEN Mrs Parker contracted smallpox, Professor Bedson and his researchers at Birmingham were working under considerable pressure. They wanted to complete their programme of research on smallpox virus by the end of 1978 when their laboratory was due to close.

The 1978 deadline for the completion of the smallpox work had been decided in 1977 when the World Health Organisation (WHO) informed Bedson that his laboratory had not been chosen as a WHO Collaborating Centre for Poxvirus Research. "It was felt", said the WHO in its letter to Bedson on 16 September, 1977 informing him of its decision, "that . . . the creation of another official centre would give the impression that WHO is itself increasing the danger of laboratory accidents. . . ." In spite of this decision, however, the WHO went on to say that the work at Birmingham was "extremely important and should be supported". It promised Bedson a \$7,500 grant for 1977.

In his reply of 4 October, Bedson described the news that his laboratory was not to become a collaborating centre as "a bit of a bombshell". The policy of the UK Department of Health and Social Security (DHSS) was that smallpox research in the UK should only go on in official WHO centres. The implication, therefore, was that Bedson's lab would have to close. This would mean that the work would now have to be done on a shortened timescale, wrote Bedson. "If pressed for a date", he went on, "I would have thought that we should aim to complete our studies with smallpox/whitepox viruses by the end of 1978".

After the WHO's response of 18 October agreeing to Bedson's plan there was a pause in correspondence until late February 1978. From then until 24 August, the day Mrs Parker's illness was diagnosed, all correspondence concerned the visit by WHO inspectors to the Birmingham laboratory on 4 May.

Before the visit took place, Bedson was eager to point out that the Birmingham facilities in no way matched those set out for the WHO's definitive smallpox labs. "It would be expensive and very costly in time", he wrote on 31 March, "if we were to try and establish such a laboratory and



quite unjustified in view of our projected halt to the smallpox/whitepox work at the end of the year". Dr I. Arita, chief of the WHO's Smallpox Eradication Unit in Geneva, assured Bedson that the expected benefit of his study "far exceeded the minimal risk" currently present in his laboratory and that the visiting team would understand his situation.

The three inspectors, however, were very critical of the safety precautions taken at the laboratory. They recommended that all mouth pipetting be prohibited, that back fastening gowns which would remain in the laboratory be worn, that hypochlorite solution be used as a permanent barrier in sinks and that gloves be worn for all work with infectious materials.

On 2 June Bedson replied to these criticisms. He accepted the suggestions concerning the use of hypochlorite solution and the wearing of gloves but argued that mouth-pipetting had not been used with smallpox for ten years. The WHO team had observed a 'tem-

porary aberration' which would not happen again whereby mouth-pipetting was used in connection with an animal poxvirus. He also argued that back fastening gowns were used in the smallpox lab and not in the animal pox lab so that they could be distinguished.

One inspector, however, was not satisfied. He was concerned about the lack of a shower, the lack of secondary containment in the animal poxvirus lab where smallpox viruses were stored and the performance and maintenance of the biological safety cabinet in the smallpox room. He recommended that the laboratory be upgraded to meet the WHO standards or "discontinue work with variola at the earliest possible date".

The next letter Bedson wrote was on 24 August when he returned from holiday. Later that day he was to find brick-shaped particles characteristic of poxviruses in vesicle fluid specimens from Mrs Parker but at the time he wrote the letter smallpox had not been

diagnosed. "There is no question", he wrote, "of our being able to upgrade our facilities to meet the full WHO standards and we are therefore proceeding with our plans to complete our studies with varola/whitepox virus by the end of this year".

What Bedson seems to have never mentioned in any of his correspondence was that the amount of smallpox work in his lab had actually increased substantially. In his letter of 2 June, he even says that there had been "a progressive decline in the scale and diversity of our operations, particularly since 1973". The Shooter inquiry found otherwise. In particular shortly after Bedson wrote this letter the rate of work increased even more as work began on 22 variola strains received from St. Mary's Hospital, London, on 26 May. Dr Mary Darlow, ex-safety officer of the Porton germ warfare establishment, commented last week: "Bedson knew he was backing a lame horse and that it would stumble sooner or later. It stumbled sooner". □

## Bedson's concern: could smallpox re-emerge?

RIFT valley fever is a cattle disease with a mortality rate of 80%. It is endemic in Egypt and recently has begun to affect man. The Egyptian authorities estimate that 18,000 people have so far caught the disease but, according to Professor Arie Zuckerman of the London School of Hygiene and Tropical Medicine (LSHTM), a team from the school which recently visited Egypt puts the number at nearer 200,000. Without suitable facilities for diagnosis and research, says Professor Zuckerman, it would be difficult to see how the spread of Rift valley fever could be prevented in Europe once the disease had crossed the Suez canal.

The situation with other diseases is similar. Early diagnosis of rabies or new diseases such as lassa fever or Marburg disease means that they can be contained. Having suitable research facilities means that vaccine and treatment can be developed in case of an outbreak.

Research is also important, however, for predicting new diseases. In the case of smallpox, for example, eradication was possible because of the existence of a very effective vaccine and because it appears to have no animal reservoir or insect vector. What worries microbiologists now, however, is that poxviruses very similar to the smallpox virus which currently affect only animals may begin to affect humans. Research on the similarities between different poxviruses and their relationship to smallpox might therefore indicate how likely this is.

The first poxvirus to be identified in a non-human primate was monkeypox virus which was isolated in 1958. Since then it has been isolated from non-human primates on several occasions and has been responsible for about 30 cases of human monkeypox in West and Central Africa between 1970 and 1978. Fortunately the spread of human monkeypox from man to man is extremely rare.

Another poxvirus very closely related to smallpox is whitepox. It was first isolated from the kidneys of cynomolgus monkeys in 1964 and it is still impossible to distinguish it from smallpox virus by laboratory tests.

The work at Birmingham centred on the identification and differentiation of poxviruses. Early work had tried to distinguish viruses by looking at the enzymes they produce. More recent work, however, had used a technique called polyacrylamide gel electrophoresis (PAGE) to separate out the polypeptide constituents of viruses and then compare them.

By mid-1978 the Birmingham team had completed comparisons of 41 different poxviruses using PAGE techniques. It had found that all but three could be put into one of four basic categories. The smallpox/whitepox category of viruses could be subdivided into types A and B. When a report of the findings was written for the WHO much more work was planned on the differentiation of types A and B smallpox viruses. Most of the B viruses seemed to be of Middle Eastern or Pakistani origin whereas the

A viruses had mainly originated in Africa. Whitepox viruses were also found to subdivide into types A and B, the B type originating in Asia and the A type in Africa. "This supports the view", said the report "that these (whitepox viruses) must be regarded as smallpox viruses".

After the extensive study of smallpox/whitepox viruses which was to have finished at the end of 1978, the Birmingham laboratory was planning to continue its study of animal pox viruses. Minor differences had been observed between some of the cowpox-like viruses and some of the monkeypox-like viruses, said the report, and those should be the subject of further study.

● The WHO says that to the best of its knowledge the only laboratories holding smallpox viruses are the following: National Institute for Virology, South Africa; Centre for Disease Control, Atlanta, USA; American Type Culture Collection, New York, USA; Walter Reed Army Institute of Research, Washington, USA; Bayerische Landesimpfanstalt, Munich, West Germany; Institut für Schiffs und Tropenkrankheiten, Hamburg, West Germany; Rijks Instituut voor de Volksgezondheid, Bilthoven, Netherlands; Laboratory of Smallpox Prophylaxis, Research Institute of Viral Preparations, Moscow, USSR; Virology Department, St Mary's Hospital Medical School, London, UK; Institute for Control of Drugs and Biological Products, Peking, China. □



# Health in the laboratory

FOR more than 20 years both government and unions have been concerned about standards of safety in British laboratories and the health of people working in them. The first major stimulus came in 1957 when D. D. Reid, of the London School of Hygiene and Tropical Medicine (LSHTM), reported that the incidence of tuberculosis among medical laboratory workers was higher than expected. As a result, a working party of the Public Health Laboratory Service (PHLS) made recommendations for safe working in laboratories handling tuberculous material. These were revised in 1968, but adverse reports continued to appear sporadically, and in 1970 the Department of Health and Social Security (DHSS) commissioned a full investigation from the Trades Union Congress (TUC) Centennial Institute of Occupational Health at LSHTM. This was carried out between 1971 and 1974 and the full report results appeared as a thesis in 1975. As far as the author is aware, the DHSS has done no more than file the report.

The investigation included retrospective surveys of the incidence of four infectious diseases and the standards of health care and safety in the laboratories of the National Health Service (NHS, consisting of hospital laboratories), the National Blood Transfusion Service and the PHLS (Harrington, J. M., & Shannon, H. S. *Brit. Med. J.* 1, 759; 1976). The surveys involved 24,000 workers, constituting 85% of the total in those laboratories. The results for the infectious diseases are summarised in Table 1.

The totals for tuberculosis in England and Wales and in Scotland represented, respectively, a 5.4 and a 4.2 times greater risk of acquiring the disease among laboratory workers than the general population. It was not possible to make a similar assessment for the other infections, but the conclusion was that hepatitis possibly remained an occupational hazard for laboratory workers, while shigellosis may have been more prevalent among them than among the general popula-

tion. All four diseases seemed most likely to afflict technicians.

Variable standards of safety and health care were reported (Harrington, J. M. & Shannon, H. S. *Brit. med. J.* 1, 626; 1977). Less than half of the laboratory workers had been examined medically before employment and a similarly small proportion had been offered vaccination. The length of service of the workers indicated that the standards had not improved during the previous 10 years and had probably deteriorated. Scottish laboratories proved to have a slightly better record in safety control than their English and Welsh counterparts, and PHLS laboratories did better than those of the NHS, providing more formal safety training.

Mouth pipetting was still being practised in 65% of English and Welsh laboratories and 28% of Scottish laboratories. Although protective clothing was usually available, its use was compulsory in only one third of English and Welsh laboratories and two-thirds of Scottish laboratories, even when procedures involved were known to be hazardous. The servicing of safety cabinets was inadequate in two-thirds of Scottish laboratories and 40% of English and Welsh laboratories, and known carcinogens were still being used in a small proportion of laboratories.

Another phase of the investigation involved a prospective survey of accidents and absence from work due to illness in 10% of the laboratories surveyed previously (Harrington, J. M. MD thesis Univ. London, 1975). Between April 1973 and March 1974 records were kept for 2,520 workers in 39 laboratories. Absence was generally lower than in other British industries. The only possible sign of a link with occupational hazards was provided by infectious and parasitic diseases, which constituted the chief cause of absence.

During this phase of the investigation 627 accidents caused injury in the 39 laboratories and one in four of the workers was affected. Technicians—64% of the population—suffered 76% of the injuries, including all nine of

those due to mouth pipetting. The most common injuries among all groups of laboratory workers were lacerations, splashes and burns to the hands or eyes, most of them relatively trivial.

The remaining phase of the investigation was a retrospective study of mortality among 156 pathologists (dying between 1955 and 1973) and 154 medical laboratory technicians (dying between 1963 and 1973) (Harrington, J. M. & Shannon, H. S. *Brit. med. J.* 4, 329; 1975). Both populations experienced lower mortality than the general population, as would be expected because any occupational group is likely to be healthier than the whole population.

The only examples of higher incidence among the laboratory populations were for lymphoma and leukaemia, which killed eight male English pathologists compared with the expected 3.3, and suicide which in England and Wales was the cause of death in nine male pathologists, compared with the expected 3.4, and nine male and six female technicians, compared with the expected 4.7 and 1.5, respectively. Suicide proved to be the commonest cause of death among English female technicians. Their easy access to poisonous chemicals was thought to be the most likely explanation. □

## Category A laboratories

**Laboratories approved by the Dangerous Pathogens Advisory Group to hold specific Category A pathogens, (in parentheses) as at December 12, 1978.**

- Department of Microbiology  
University of Reading (Rabies)
- Central Public Health Laboratory  
Colindale (Rabies)
- Central Veterinary Laboratory  
Weybridge, Surrey (Rabies)
- Evans Biologists Ltd  
Liverpool (Rabies)
- Animal Virus Research Institute  
Pirbright (Rabies)
- Lister Institute  
Elstree (Rabies)
- Institute of Virology  
University of Glasgow (Rabies)
- Microbiological Research Establishment  
Porton, Wilts. (Rabies, Lassa fever,  
Marburg, Simian herpes B, Crimean  
haemorrhagic fever (Congo),  
Venezuelan equine encephalitis, Ebola)
- London School of Hygiene and  
Tropical Medicine  
Winches Farm, St Albans (Crimean  
haemorrhagic fever (Congo), Venezuelan  
equine encephalitis)
- Department of Virology  
St Mary's Hospital Medical School  
London (Smallpox)
- Wellcome Research Laboratories Ltd  
Beckenham, Kent (Rabies)

**Table 1 Infectious diseases in laboratory workers in England and Wales in 1971 and Scotland in 1973.**

| Infection    | Medical staff | Scientific staff | Technical staff | Other | Total  |
|--------------|---------------|------------------|-----------------|-------|--------|
| Tuberculosis | 1 (1)         | 1                | 14 (2)          | 2     | 18 (3) |
| Hepatitis    | 2 (1)         | 1                | 27 (2)          | 5     | 35 (3) |
| Shigellosis  | 1             | 3 (1)            | 29 (7)          | 4     | 37 (8) |
| Brucellosis  | —             | —                | 1               | —     | 1      |

Figures for Scotland are in parentheses.

American Association for the Advancement of Science meeting in Houston, Texas.

Reports by David Dickson

## AAAS president calls for closer university/industry links

WITH US industry under pressure to remain competitive, and universities facing problems in securing research support from conventional sources, the time has come for a closer relationship between the two, according to Dr Edward David, president of the American Association for the Advancement of Science.

Close links between the industrial and academic communities could help to preserve important features of the research system: a pluralism of funding sources, leading to greater local autonomy in the conduct of research and development programmes; a more competitive environment, based on technical excellence; and a dependence on "high achievers" able

to provide scientific leadership.

If basic research was defined not as research carried out with no particular goal in mind, but rather as any research leading to a greater understanding of natural and social processes, regardless of the motive, then the amount of such research being carried out by industry was larger than current definitions indicated. Industry had also widened its research interests beyond just improving the technology of production lines. "Today there is a much broader point of view, ranging from the sociology of customers, to research on the R&D process itself." Industrial research was increasingly using the basic research paradigm in forwarding its function,

and it would not be incompatible to couple this directly with academic interests.

In the 1950s and 1960s, industry support for university research had been primarily philanthropic in nature. Now a number of companies were exploring ways of setting up long-term cooperative arrangements with universities. "Ten years from now I expect to see a vigorous community of industrial/academic scientists, with academics providing the ideas and industry providing the vehicle for their commercialisation. This will certainly be an improvement on the situation that we have now. And it will be more in keeping with the situation facing both sides in the 1980s." □

## US to brief scientists on human rights

THE US State Department is preparing briefing materials for scientists and others who participate in official exchanges, describing the goals and objectives of the US human rights policy, and responding to questions about the legality and safety of activities in defence of colleagues abroad.

Mrs Roberta Cohen, of the Department's Bureau of Human Rights and Humanitarian Affairs, told a session on international freedom and responsibility that US Government agencies and representatives had been instructed not to discourage or prohibit exchange participants from contacting private Soviet citizens. "We in the Bureau of Human Rights take it as an obligation of the Government to correct any wrong impression created that it is seeking to discourage scientists on exchange programmes from efforts to defend foreign colleagues," she said.

The Government asked only that contacts of potential sensitivity, particularly in tense periods, be discussed in advance with Administration officials, "in order to ensure that American scientists involved have full information assessing the potential risks".

A blanket halt to exchanges had distinct disadvantages, ranging from a reduction in the exchange of ideas to raising the spectre of Government interference. Boycotts of meetings were not likely to be as productive as participating and expressing human rights concerns, she said. □

## Rethink for research funding

A LARGE proportion of federal funds for university research should be distributed to institutions on a "formula" basis calculated on proven capability and productivity. Such a "deregulated and decentralised" system for research grants would avoid much of the unnecessary work involved in the current grant - proposal/peer - review process, Professor Rustum Roy, chairman of the science, technology and society programme at Pennsylvania State University, told a session on federal research and development funding. He also suggested a three-year national commission to set national priorities in research and development, with a strong input from the scientific and engineering community.

Professor Roy said existing research and development mechanisms were designed for the "golden age" of science in the 1950s and early 1960s, but were no longer appropriate. The new social setting for R and D included: public recognition of the "ambivalence" of science and technology, rather than seeing either as solely beneficial; widening gap between science and the public; a recognition that there is no simple relationship between support for basic science and a healthy economy; and the fact that the system had not led to maximum innovation in the industrial sector.

The problem lay not so much in getting more money for basic science—"possibly the least important question"—as in designing a more efficient system for the disbursement of funds, where

radical changes could produce a major increase in productivity.

A clearly stated rationale for the support of science was also needed. Most important would be contributions to nationally-defined objectives such as defence, health and energy; then would follow science for graduate education, science for state, community and local needs, and finally "science for science's sake". Congressman George E. Brown, a leading member of the House of Representatives Committee on Science and Technology, pleaded for a more sophisticated strategic planning operation for the national scientific effort.

"It is impossible to develop a new rationale for the support of science and better mechanisms for carrying this out without a strong and well-defined central planning authority in the scientific sphere," he said. Some members of Congress had hoped that this role would be played by the President's Office of Science and Technology Policy when it was reestablished in 1976. However, many of the policy and planning tasks envisioned for OSTP had gone to other offices further from the executive centre.

Another need was to develop mutually reinforcing networks of research institutions. "The capabilities of various federal, state and local laboratories must be better linked, both in terms of facilities and people. Only when we have taken such moves can we persuade the public and Congress that critical gaps in research remain and are in need of support." □



## Assessment of cancer risks comes under attack

EXTRAPOLATING downwards from the effects of exposure to high levels of ionising radiation is an inadequate basis for predicting the dose-response relationship for the incidence of cancer at low levels of exposure, according to Dr Alice Stewart, of the Department of Social Medicine at Birmingham University, England. Such extrapolation would only be valid if cancer was the sole effect of radiation. However there were other indirect effects which would be hidden by the high-dose data, but which would make a linear dose-response relationship untenable, Dr Stewart told a session on epidemiology studies of low-level radiation exposure.

Recent analysis of medical data collected on workers at the US Government's nuclear facilities at Hanford in Washington State, and therefore based solely on low levels of exposure, indicated that a curvilinear model for the dose-response relationship was better than a linear model. The best fit was given by a curve in which the response was proportional to the square root of the dose.

Dr Stewart said medical considerations raised questions about using data based on survivors of the atomic bomb explosions at Hiroshima and Nagasaki as a basis for predicting the effects of low-level exposure to ionising radiation. "Claims that the mortality of A-bomb

survivors between 1950 and 1974 from diseases other than cancer is unrelated to ionising radiation gave a false impression. This was due partly to the fact that direct and indirect effects of radiation delivered at high dose-rates had similar consequences, and partly to the ease with which other diseases could prevent cancer induction by radiation—or other carcinogens—from developing into clinical cancer".

Prevention at this stage was usually the result of a non-cancer death coinciding with a period of cancer latency. "Optimal conditions for observing cancer effects of radiation are therefore those which combined low rates of general mortality with small doses of radiation delivered at slow dose rates."

Dr Stewart said that these were the conditions surrounding data collected by Dr Thomas Mancuso, of the University of Pittsburgh, on the health of workers at the Hanford plant, which have recently caused considerable controversy by raising serious doubts about the adequacy of current protection measures.

Mr George Kneale, a Birmingham University statistician who has been helping Dr Stewart to analyse Dr Mancuso's results, told the session that the most recent analysis of the data provided a doubling-dose for the

incidence of radiation-linked cancers of between 2 and 150 rads, whereas calculations based on a linear extrapolation from data on A-bomb survivors indicated a doubling-dose of between 150 and 200 rads. "On internal evidence the Hanford survey rejects the possibility that the true risk may be as low as it seems from the A-bomb survivors, but we cannot say precisely what the true risk is, apart from the fact that it appears to be higher at low doses than extrapolation downwards from high doses would indicate."

Doubts about the validity of conclusions of epidemiological surveys based on relatively small sample data were raised by Dr Charles E. Land, of the National Cancer Institute's environmental epidemiology branch. Dr Land warned that an inadequate sample size severely reduced the chances of correctly rejecting a false hypothesis of no dose effect at low levels of exposure. "Failure to reject is not strong evidence for the null hypothesis, so the study results are likely to be inconclusive—a wasted effort", he said.

"We do not have the resources for adequate epidemiological studies of populations exposed to low levels of radiation, and if we should try to do them anyway we would run considerable risk of obtaining misleading results, results that would derive at least some credibility from the vast effort of obtaining them," he said. □

Meanwhile, back in Washington . . .

## DNA critics appointed to advisory committee

Two active participants in recent debates over the adequacy of guidelines for research using recombinant DNA techniques have been appointed to the committee responsible for advising the director of the National Institutes of Health on a number of important aspects of such research.

The two—Dr Richard Goldstein, assistant professor of microbiology and molecular genetics at Harvard Medical School, and Dr Sheldon Krimsky, acting director of the program in urban, social and environmental policy at Tufts University—are among 14 new members appointed to the Department of Health, Education and Welfare's Recombinant DNA Advisory Committee by HEW Secretary Joseph A. Califano.

The appointments follow Mr Califano's announcement last month of a number of changes to existing arrangements for regulating recombinant DNA research. The advisory committee is responsible for giving advice on new types of bacteria for use in

such research, on whether certain presently prohibited experiments should be conducted, whether additional categories of research should be exempted from the guidelines, and on possible future changes in the guidelines.

Mr Califano also announced that the size of the advisory committee was to be increased to 25, and that the scope of its membership was to be extended to give greater weight to non-scientific representation. The new members of the committee include a professor of education, a professor of law, a prominent environmentalist and a laboratory technician.

In addition to Dr Goldstein and Dr Krimsky, the new members include: Dr Karim Ahmed, senior staff scientist with the Natural Resources Defense Council; Zelma Cason, chief of cytotechnology in the department of cytology at the University of Mississippi Medical Center; Patricia King, professor of law at Georgetown University Law Center in Washington; Dr

Samuel Proctor, professor of education at Rutgers University; and Ray Thornton, retiring member of the US House of Representatives and chairman of the House subcommittee on science, research and technology in the last Congress.

The new scientific and medical members are: Dr David Baltimore, (MIT); Dr Francis Broadbent (University of California, Davis); Dr Richard Novick (New York Public Health Research Institute); Dr David Parkinson, (University of Pittsburgh); Dr Damon Pinon, (University of California); Dr Luther Williams (Purdue University); and Dr Frank Young (University of Rochester).

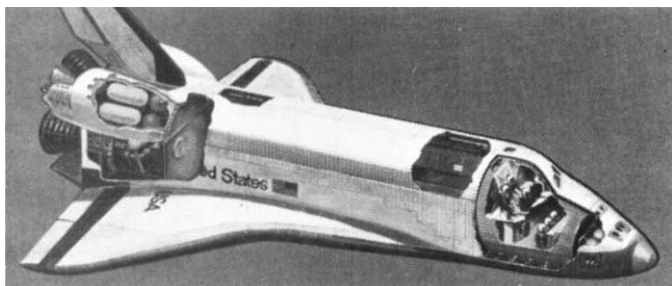
There is thought to have been considerable controversy over some of the other names of "public interest" representatives suggested for possible membership of the committee, particularly since some scientists involved in the research felt that too large a non-technical component might impede the committee's functioning. □



# news in brief

**Engine failure threatens further delay to space shuttle:** An engine failure during testing is threatening further delays to the maiden flight of the US National Aeronautics and Space Administration's space shuttle (artist's impression below), currently scheduled for 28 September. The failure occurred during tests two weeks ago near St. Louis, Missouri, when a fire in the high pressure turbo-pump that feeds oxygen into the combustion chamber caused the engine to explode.

Space shuttle's initial flight has already been postponed from March following other problems in the engine development programme, and the delays have added extra costs to the programme. According to NASA, the test engine was heavily damaged in the explosion, and it is expected that it will take three to four weeks to repair the test stand. It is not yet known whether the engine failure will affect the current launching schedule. The agency issued a statement last week saying that an assessment of the impact to the engine test programme and to the first flight schedule was now being made.



**Argon isotope ratio on Venus differs from Earth:** Data from the latest Soviet Venus probes, Venera-11 and -12, which reached the planet at the end of December "astounded everyone"—according to a report from the flight control centre, broadcast a few hours after the second descent module had landed. The surprising result, it appears, was the unexpectedly high ratio of  $^{36}\text{Ar}$  to  $^{40}\text{Ar}$  in the atmosphere—some 200–300 times higher than the ratio of argon isotopes in the terrestrial atmosphere.

Perhaps the most surprising thing about the result, however, is that this value is a close confirmation of the result obtained by the latest US probe which reached Venus some three weeks before the Soviet modules. If the flight control centre was indeed "astounded", this surely reflects on the rate at which the Soviet team get access to the data from US missions—a point which could well be taken up in any future discussions of peaceful cooperation in space research.

According to the US researchers, the new data suggests a two-stage accretion process of planet forming. Professor Zolotukhin, Deputy Director of the Space Research Institute of the Soviet Academy of Sciences, seems to confirm this view, saying that the Venus argon ratio is "important for the study of the evolution of the planet".

A more popular report from the flight control centre, however, took a somewhat different line. According to commentator Nikolai Zheleznov, the argon value, taken in conjunction with the high surface temperature and dense atmosphere, suggests that the process of chemical evolution of the substances comprising the planet was "lengthened", thereby making the "paths of development" of Earth and Venus "diverge".

**UK scientist wins redundancy pay:** A cell biologist has won £400 redundancy pay from his former employer, the Imperial Cancer Research Fund. The ICRF has a clause in its contracts of employment for untenured staff which states

that they waive their rights to redundancy payment. Redundancy claims therefore are complicated and must be based on technicalities in the law. In this case the contract was for one year. The waiver clause is invalid for contracts of less than two years. The ICRF could have argued that the scientist's employment, consisting of one two-year contract and two one-year contracts, constituted more than two years total employment but decided not to contest the claim. Workers under age 41 are entitled to one week's pay for each year of employment not exceeding £100 per week. Of the ICRF's 128 scientific staff 40% are tenured, 15% on limited-term contracts are eligible for tenure and 45% are employed on non-renewable fellowships.

**Sun powered telephones in Jordan:** Over 80 solar powered telephones have been installed along Jordan's roads to provide motorists with an emergency telephone service. At the top of each telephone pole is a plate of solar cells which convert sunlight into an electric current that charges a storage battery of 36-hour capacity. The telephone is connected to a UHF transmitter powerful enough to reach the nearest microwave installation. Each telephone is equipped with a series of push buttons marked for the type of emergency service needed. At the push of a button the corresponding signal is relayed to an operator at one of three central exchanges. The system is especially designed for foreign speakers but a receiver is available for voice communication.

**Saudi Arabia creates a new science and technology centre:** The Kingdom of Saudi Arabia has announced the formation of an independent Saudi National Centre of Science and Technology. The centre will be responsible for support of scientific research and for coordination of existing scientific agencies in accordance with Saudi Arabian Development Plans. The brief of the new centre includes starting special research laboratories appropriate to the research needs of the kingdom, assistance to the private sector for research into increasing industrial and agricultural production, and support for specific research projects including grants to develop local skills for scientific research. The centre is to seek joint projects with international scientific agencies and to propose a detailed plan "to realise the kingdom's objectives in technological fields". The local scientific community has welcomed the centre as an important part of Saudi Arabian social and economic development.

**EEC announces fusion programme for 1979–1983:** The total cost of the 1979–83 fusion programme proposed by the European Commission is 921 million European units of account (1 EUA=\$1.3). Of this sum 185 million EUA is scheduled for the Joint European Torus (JET) of which 80% will be paid by the commission. The remaining 736 million EUA will be spent over the next five years on the rest of the EEC's fusion programme which includes research on Tokamaks, new plasma heating systems, inertial confinement, tritium technology and materials research.

Already 70% of the contracts have been placed for the fabrication of the machine parts of JET. In February the first stone will be laid for its building in Culham near Oxford. Construction of the machine itself will begin in 1981 and is expected to be finished by 1983 when the first tests can begin. JET is to be built with the participation of all nine community countries plus Sweden and (shortly) Switzerland. After a three-year struggle, the Council of Ministers decided in May 1978 to site the machine in the UK at Culham.

# news and views

## Amorphous silicon: where now?

from S. R. Elliott

ONE of the more extensively studied materials in solid-state research at present, certainly in the field of amorphous semiconductors, is the amorphous silicon-hydrogen alloy system. Upward of 30 laboratories around the world are now working on these materials. The reasons for all this activity are, of course, not hard to discern; they are principally its photovoltaic properties and their possible exploitation in solar energy conversion (see for example *News and Views* 275, 93; 1978). The attraction lies chiefly in the fact that large area, thin film solar cells could be made relatively cheaply (particularly compared with conventional single crystal devices). However, at present amorphous devices can manage at best only  $\approx 6\%$  conversion efficiency (in contrast to crystalline Si devices that reach  $\approx 16\%$ ), and it seems that such limitations could be inherent to the process which is currently used to produce these amorphous silicon (a-Si) films.

Spear and his co-workers at the University of Dundee announced in 1975 that by producing a-Si films by means of the decomposition of silane ( $\text{SiH}_4$ ) using an r.f. glow-discharge, doping could easily be achieved by admitting phosphine ( $\text{PH}_3$ ) or diborane ( $\text{B}_2\text{H}_6$ ) to the gas flow producing samples which were n-type (the majority current carriers being electrons) or p-type (the majority carriers being holes), respectively. This offered many prospective applications using for example p-n junctions, and was a very significant advance over previous methods of producing a-Si (by vacuum evaporation or sputtering in argon), in which doping was not achieved upon incorporation of the appropriate impurities. The broken (or dangling) bonds introduced in large densities ( $\approx 10^{19} \text{ cm}^{-3}$ ) by these latter preparative techniques give rise to electron states lying deep within the forbidden energy gap between valence

and conduction bands, which can trap any excess carriers introduced by dopant atoms. Indeed, these defect states control to a very large extent much of the electronic behaviour of such films.

Studies by Fritzsche (University of Chicago) and Brodsky (at IBM) and others have indicated that a relatively high concentration of hydrogen ( $\approx 5\text{--}10 \text{ at.}\%$ , sometimes more) remains within the films prepared by glow-discharge decomposition, a finding not altogether surprising in view of the fact that the decomposition of silane is thermodynamically endothermic. It therefore appears that the presence of hydrogen is in some sense essential to the favourable properties of glow-discharge a-Si. Naively, this can be explained by the notion of hydrogen bonding to the dangling Si bonds, thereby removing their electron states from the energy gap, and thus making doping possible. However, this requires at most  $\approx 0.1 \text{ at.}\%$  hydrogen, far less than the amount that is found experimentally. It must be assumed that the balance of the hydrogen is bonded into the random network during growth. The importance of hydrogen in producing films that have favourable photovoltaic properties is underlined by the work of Paul and coworkers at Harvard University, who have produced a-Si:H alloys by sputtering in an atmosphere comprising both argon and hydrogen, which can be doped, and which therefore behave in a manner somewhat similar to those produced by the glow-discharge decomposition of silane.

Despite the promising progress made so far in the field of photovoltaic applications, several problems remain with glow-discharge-produced a-Si. Numbered amongst these are: (1) the variation in properties with the conditions of deposition (for example, the hydrogen content, and hence allied properties, is a sensitive function of substrate temperature, gas pressure, r.f. power); (2) photo-induced changes, such as the metastable decrease in conductivity after optical illumination

(Staebler & Wronski *Appl. Phys. Lett.* 31, 292; 1977); (3) the relative inefficiency of doping, in that only  $\approx 30\%$  of impurity atoms act as donors or acceptors (that is  $\approx 70\%$  bond according to their normal valence and are not forced into tetrahedral environments on substituting for Si atoms) and the Fermi level can only be moved to within  $\approx 0.2 \text{ eV}$  of the conduction or valence band edge; and (4) short minority-carrier lifetimes. In this context, a recent publication by the Harvard group (Paesler *et al. Phys. Rev. Lett.* 41, 1942; 1978) entitled 'New development in the study of a-Si:H alloys: the story of O', is of interest. This group concentrates on material prepared by sputtering in partial hydrogen atmospheres. They point out that many of the differences in behaviour exhibited by these alloys, compared with those prepared by the glow-discharge decomposition of silane, could be ascribed to the presence of oxygen in the latter; the poorer vacuum and often less pure gases used in the glow-discharge process immediately imply this. To test this hypothesis, samples were produced by sputtering in an argon atmosphere containing both hydrogen and oxygen; the resulting films contained  $\approx 1 \text{ at.}\%$  oxygen. They found that the oxygenated films exhibited a much larger photoconductivity and photoluminescence and were relatively insensitive to the partial pressure of hydrogen used, in marked contrast to oxygen-free films; in short, the oxygenated sputtered films more closely resembled glow-discharge samples. It was concluded that in some way the presence of oxygen (along with hydrogen) removes non-radiative recombination channels, perhaps by saturating any dangling bonds present.

Evidence was also presented for the importance of structural defects in the photoluminescence process. This is a much debated subject at present; controversy centres on whether electron states in the energy gap of a-Si:H alloys are introduced by hydrogen atoms attached to Si atoms other than

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by simple covalent bonds (Appelbaum *et al. Phys. Rev. Lett.* **39**, 1487; 1977; Singh *et al. Phys. Stat. Sol. (b)* **81**, 637; 1977; Fisch & Licciardello *Phys. Rev. Lett.* **41**, 889; 1978), or whether simple reconstructed Si dangling bonds containing either one or two electrons (and hence positively or negatively charged respectively), give rise to such states, as proposed by the author and Adler (to appear in *Philos. Mag. and Phys. Rev. Lett.*, respectively). The short minority carrier lifetime, one contribution to poor device performance, can for example be attributed to such states within the gap, of whatever origin. The Harvard group's paper indicates a possible solution to the problem of the nature of the defects,

although it does not resolve the issue.

However, all such considerations may be made redundant with the announcement by Ovshinsky (*Nature* **276**, 482; 1978) that a-Si alloys produced from a mixture of silicon tetrafluoride (SiF<sub>4</sub>) and hydrogen, and therefore containing both fluorine and hydrogen, are markedly superior to those produced by the glow-discharge decomposition of silane. Doping efficiencies are larger, photostructural changes are absent, and deposition conditions only weakly affect the properties of these a-Si-F-H alloys, in sharp contrast to the problems that bedevil silane glow-discharge films. Perhaps here lies the road ahead for amorphous silicon alloys. □

**B279**, 81; 1977 and in *Adaptations Within Antarctic Ecosystems* (ed. Llano), Smithsonian Institution, 1977, hereafter referred to as Laws I and II, respectively) has drawn together much earlier work, to give a fascinating account of the mechanisms of ecological separation among these whale and other species.

The standing crop of zooplankton, mainly krill, in the Antarctic is at least four times that in the tropics, but it is a highly seasonal resource for whales. In addition to seasonal changes in krill abundance itself, the krill zone is nearly all covered by pack ice in the southern winter; in summer the ice retreats and 80–90% of the krill zone is exposed when the pack ice is at its minimum in March. Most of the baleen whales make long migrations, feeding in summer in the food-rich Antarctic waters, and breeding in winter at low latitudes, where they of necessity fast and rely on energy stored as fat (blubber). A conservative estimate of the weight lost in winter is 20%, which compares with many terrestrial mammals, such as deer. The migrations appear to be staggered, with the different species arriving in the Antarctic at different times in the summer. The blue appears earliest, the fin and humpback next, and then the sei. These waves of migration also reflect the extent to which the different species penetrate into colder waters; the blue appears first and follows the retreating pack ice south, while the other species fill in behind it. Ohsumi *et al. (Sci. Rep. Whales Res. Inst.* **22**, 75; 1970) estimate that the highest densities of sei whales are found in the circumpolar zone spanning 40–50°S, fin in 50–60°S, and blue and minke in 60–70°S. The pigmy blue whale seems confined to waters north of 54°S. The southern right whale is probably not migratory and may feed all year in the zone 30–50°S (see Fig. 1).

With the conspicuous exception of the minke, the timing and extent of the migration patterns correlate with the body sizes of the species, and with their percentage proportions of meat and blubber: blue, 39% meat and 27% blubber; fin, 46% and 24%; sei, 59% and 17%; minke, 62% meat and 15% blubber. Although the minke penetrates far south, its low proportion of blubber and small size suggest it is a more continuous feeder than the larger and more migratory species. It also has a higher population turnover, with a 1-year reproductive cycle in contrast to the other whales' 2-year cycle. Within this set of species, we may view the minke as the r-selected opportunist, superimposed on the smooth pattern of spatial and temporal segregation manifested by the other baleen whales.

The baleen whales are the top pre-

## Ecological interactions in the Southern Ocean

from Robert M. May

THE Southern Ocean sustains a great variety of vertebrate species, including whales, seals, birds and fish.

Very many of these species share a common resource, the shrimp-like krill, as the main component of their diet. The mechanisms whereby these competing species coexist are of interest in illuminating general ecological principles. Viewed in this light, the massive depletion of the populations of large whales constitutes a vast, and sadly ill-documented, ecological experiment.

### Whales

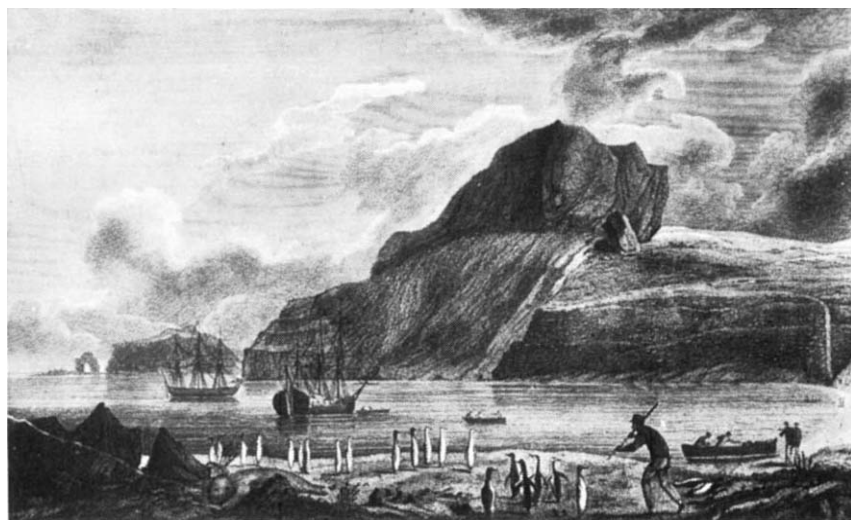
As a group, the whales probably origin-

ated about 60 million years ago. Their patterns of distribution in the Southern Ocean must have been subject to many changes in the past few hundred thousand years, with the comings and goings of the Ice Ages. The large baleen whales in this area comprise six species and one subspecies—the southern right, humpback, fin, sei, minke, blue, and the subspecies pigmy blue. There is one large toothed whale, the sperm; only adult males enter Antarctic waters. In addition there are nine species of small toothed whales, which include the killer whale *Orcinus orca*, the southern white-sided dolphin, and two species of beaked whales. Little is known about the ecology of these smaller toothed whales.

Laws (*Phil. Trans. Roy. Soc. Lond.*

Robert M. May is Class of 1877 Professor of Zoology at Princeton University.

*A view of Christmas Harbour in Kerguelen Land. From a contemporary account of Cook's third voyage.*



dators in a very short food chain, filter-feeding on planktonic crustacea. The rough geographical zones occupied by krill, the major species of these crustacea, are also indicated in Fig. 1. The baleen plates of the various whales are adapted to the size of the food taken: the right whale, which feeds primarily on small copepods, has very fine long baleen and the mouth is enlarged to accommodate them; the filtration by the plates of the sei, minke, humpback, fin and blue is increasingly coarse. Feeding behaviour also varies. The fast-swimming blue, fin, minke and humpback whales swallow in discrete mouthfuls which is more effective with swarming food organisms such as krill, while skimming with the mouth closing at longer intervals (as done by right whales) works better against dispersed organisms such as copepods. The sei uses a combination of the two.

The main sperm whale stocks are found outside Antarctic waters, and females rarely penetrate latitudes beyond 45°S. Solitary males begin to show migratory behaviour as they attain sexual maturity (starting around age 12 years), and their propensity to migrate to Antarctic waters in the summer increases as they get older, levelling off around age 25 years when they may attain the status of harem master in the breeding herds at low latitudes. Sperm whales feed largely on squid species found in temperate and Antarctic waters.

Although not much is known about them, the smaller toothed whales are probably resident in Antarctic seas all the year round.

Rough estimates of the past and present numbers and biomass of large whales in the Antarctic are given in Tables 1 and 2, along with estimates of their food consumption (simplified from Laws I). These numbers are not graven in stone; the many uncertainties in the data are such that no two published tables are identical. A list of the main assumptions and uncertainties is as follows. One-half the total sei whale stock, and one-third of the 'exploitable male' sperm whales are assumed to feed in the Antarctic, south of the Antarctic Convergence. The figure of 10,000 blue whales is higher than the 1978 IWC figures, as is their mean weight of 83 tonnes; Masaki and Yamamura (*Rep. int. Whal. Commn* 28, 258; 1978) give 4,500 at an average weight of 62 tonnes. The minke figures, both past and present, are very uncertain, and estimates range widely (the minke have only recently been exploited, and most information derives from commercial whaling). With the exception of the minke, which is assumed to feed all year in Antarctic seas, the whales are taken to spend

Table 1 Initial whale stocks

| Species       | Population<br>(thousands) | Weight<br>(thousand<br>tonnes) | Biomass<br>(million<br>tonnes) | Food consumed<br>(million tonnes) |       |      |
|---------------|---------------------------|--------------------------------|--------------------------------|-----------------------------------|-------|------|
|               |                           |                                |                                | Krill                             | Squid | Fish |
| Baleen whales |                           |                                |                                |                                   |       |      |
| Blue          | 200                       | 88                             | 17.6                           | 72                                | 0.74  | 1.5  |
| Fin           | 400                       | 50                             | 20.0                           | 81                                | 0.84  | 1.7  |
| Humpback      | 100                       | 27                             | 2.7                            | 11                                | 1.11  | 0.2  |
| Sei           | 75                        | 19                             | 1.4                            | 6                                 | 0.06  | 0.1  |
| Minke         | 200                       | 7                              | 1.4                            | 20                                | 0.20  | 0.4  |
| Baleen total  | 975                       | —                              | 43.1                           | 190                               | 2.00  | 3.9  |
| Sperm whale   | 85                        | 30                             | 2.6                            | —                                 | 10.2  | 0.5  |

Table 2 Present whale stocks

| Species       | Population<br>(thousands) | Weight<br>(thousand<br>tonnes) | Biomass<br>(million<br>tonnes) | Food consumed<br>(million tonnes) |       |      |
|---------------|---------------------------|--------------------------------|--------------------------------|-----------------------------------|-------|------|
|               |                           |                                |                                | Krill                             | Squid | Fish |
| Baleen whales |                           |                                |                                |                                   |       |      |
| Blue          | 10                        | 83                             | 0.83                           | 3.4                               | 0.04  | 0.07 |
| Fin           | 84                        | 48                             | 4.03                           | 16.4                              | 0.17  | 0.34 |
| Humpback      | 3                         | 27                             | 0.08                           | 0.3                               | 0.00  | 0.00 |
| Sei           | 41                        | 18                             | 0.71                           | 2.9                               | 0.03  | 0.06 |
| Minke         | 200                       | 7                              | 1.40                           | 19.8                              | 0.20  | 0.41 |
| Baleen total  | 338                       | —                              | 7.05                           | 42.8                              | 0.44  | 0.88 |
| Sperm whale   | 43                        | 27                             | 1.16                           | —                                 | 4.63  | 0.24 |

120 days feeding in the Antarctic.

Some of the consequences of the great differences between initial and present stocks are pursued below. A gross summary is that the pristine biomass density of baleen whales over the area of the krill zone was about 2.6 g m<sup>-2</sup>, while it is now 0.4 g m<sup>-2</sup>.

### Seals

The seal families probably originated about 30 million years ago in the northern hemisphere, and crossed the tropics along the west coast of South America during a cold period, to radiate into the Southern Ocean. There are now five species of 'true' seals south of the Antarctic Convergence—Weddell, leopard, crabeater, Ross and elephant—along with the Antarctic fur seal. Although there are fewer species in the Antarctic than in the Arctic (probably because the wide deep ocean has no geographical barriers to help speciation), the populations of individual species and the body sizes of their constituent members are larger (probably because food is more abundant). Of the roughly 30 million seals in the world, approximately half live in the Antarctic, where they constitute about 80% of the global biomass of seals.

The species achieve ecological segregation through a mixture of different habitats and different diets. The geographical aspect is set out schematically in Fig. 1, and the food preferences summarised in Table 3. The elephant and Ross seals are both deep-diving squid and fish eaters, but their breeding and feeding distributions have

no overlap; the elephant seal breeds on land and is distributed about the Antarctic Convergence, while the Ross seal breeds on ice and is found near heavy pack ice. The fur and crabeater seals are primarily krill eaters; its name notwithstanding, the crabeater has dentition specialised to act as a krill-sieve, producing "perhaps the most complicated arrangement of cusps found in any living mammal" (Laws II). The fur seal breeds on land, and is restricted to the area between the pack ice edge and the Antarctic Convergence, while the crabeater breeds on ice and is restricted to the fringes of the pack ice belt. The leopard seal has a more varied diet (krill, fish, and seasonally penguins) and a wider range than the other seals, but it is primarily found at the fringes of the pack ice zone. The Weddell seal has adapted to fill a specialised niche—the inshore fast ice zone—where it winters under the ice, keeping breathing holes open with its teeth. It eats mainly fish.

The mating systems and patterns of sexual dimorphism correlate strongly with the ecology of the Antarctic seals (Stirling *Rapp. P.-v. Renn. Cons. int. Explor. Mer.* 169, 205; 1975). The polygynous Weddell has a social system intermediate between the highly polygynous and strongly dimorphic elephant and fur seals that breed on land, and the monogamous crabeater and other species that breed on shifting pack ice.

From Tables 1-3 it can be seen that the total biomass of Antarctic seals is less than 10% of the original biomass of large Antarctic whales, and less than



half their present biomass. But the seals are present in Antarctic waters all year, and furthermore their metabolic rates and food consumption are relatively larger, because of their smaller body sizes. The consequent estimates of their total annual consumption of krill is about one-third that of the original stocks of baleen whales, and about 1.5 times that of the present whale stocks; seals have now surpassed whales as the major consumers of krill. Essentially all the krill eaten by seals is by crabeaters (to an excellent approximation all Antarctic seals are crabeater seals), who, by foraging at the margins of the pack ice, have relatively little direct overlap with whales.

Male sperm whales and both sexes of elephant seals eat mainly squid in the Antarctic, and, at present whale densities, they eat roughly equal amounts. However, when the sperm whales are at peak abundance a large fraction of the elephant seal population is hauled out on land, fasting while breeding and moulting.

The average biomass density of krill-eating seals in summer is around  $0.6 \text{ g m}^{-2}$ , in regions where they compete with fish, birds and minke whales. The winter density is probably similar, but spread over a different area. Squid and fish-eating seals reach densities around  $0.1 \text{ g m}^{-2}$  when concentrated in summer, and around  $0.03 \text{ g m}^{-2}$  when dispersed in winter.

### Birds and fish

Surveying a growing, but still limited, amount of information, Laws (II) concludes "the ecological separation of Antarctic birds appears to be determined by the summer distribution and breeding behaviour as they affect feeding". The segregation in the distribution and feeding ranges of some key species of penguins and other birds are shown in Fig. 1.

One example of the way the species sort themselves out is provided by three species of penguin that breed at Signy Island. The Adelie and chinstrap penguins feed mainly on krill, but they take different sized prey, either by active selection or by the Adelie feed-

ing further from the rookery (or penguinery). The less common gentoo penguin feeds primarily on fish taken from the seabed.

Petrels and albatrosses also eat krill, squid and fish. In general, the lower latitude species such as the albatrosses are more dependent on squid and fish than the purely Antarctic species.

It is difficult to quantify the biomass and role in the ecosystem of Antarctic birds. Laws gives a rough estimate of  $0.03 \text{ g m}^{-2}$  for their biomass density when concentrated in the feeding zone in summer, and no more than  $0.004 \text{ g m}^{-2}$  when dispersed in winter.

Although the facts are not well established, the fish stocks in the Antarctic are probably much less than those in the Arctic. The main reason is that the Antarctic has a very narrow and relatively deep continental shelf, so that spawning sites for bottom-spawning fish are very limited. Some southern species have overcome this problem by spawning in coastal waters in the Subantarctic or on the continental shelf of Patagonia, and assuming a pelagic life as adults (when they feed on krill). In contrast with the north, where fish are the main vertebrate animals, in the Southern Ocean whales, seals and birds are more important.

### Responses to the decrease in whales

By comparing Tables 1 and 2, it can be seen that the difference between the krill consumed annually by the original stocks of whales and that consumed today amounts to around 150 million tonnes.

What are the implications of this 'krill surplus' produced by whaling? General ecological theory suggests that, in the extreme case where there is no competition among whale species, nor between whales and other species, the 'surplus' will indeed appear as more krill. At the opposite extreme of high niche overlap among the vertebrate species, most of this 150 million tonnes of 'slack in the system' will be taken up (with time lags that may be measured in decades), causing shifts in the relative abundances of species. I think the reality is liable to lie between these two extremes.

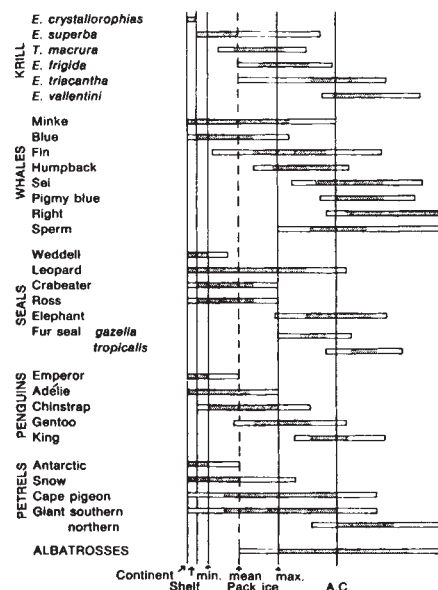


Fig. 1 A comparison of the zones occupied by selected species of krill, marine mammals and birds from the edge of the Antarctic Continent northward. The relative areas of the continental shelf, the minimum, mean and maximum area of pack ice, and of the region south of the Antarctic Convergence (A.C.) are as shown. Each species has a circum-polar distribution, and the average latitudinal range is as shown, with the shading denoting the regime of higher densities. (From Laws II).

Laws (I and II) summarises direct and indirect evidence of population increases in whales, seals and birds that may be attributed to the decline in baleen whale populations.

Increases in the numbers of king penguins seem greater than can be explained simply as recovery from the slaughter of the nineteenth century. Population increases have also been shown for chinstrap, Adelie and gentoo penguins. It is noteworthy that the best-documented increases in these populations of krill-eating penguins are in the South Atlantic, where there is the greatest overlap between whales and penguins. In contrast, there is no indication of population increase for Adelie penguins on Ross Island over the past 50 years; interaction with whales is arguably small in the Ross Sea.

Payne (*Phil. Trans. Roy. Soc. Lond.* B279, 67; 1977) has established increases in the populations of the southern fur seal that clearly run ahead of simple recovery from over-exploitation. The fastest increase for this krill-feeding animal have taken place in the Scotia Arc, where its distribution overlaps that of baleen whales.

The sei whale has in recent years altered its migration patterns, arriving in the Antarctic earlier and penetrating further south. This could be due to

Table 3 Seals

| Species   | Population<br>(thousands) | Weight<br>(thousand tonnes) | Biomass<br>(million tonnes) | Food consumed<br>(million tonnes) |       |      |
|-----------|---------------------------|-----------------------------|-----------------------------|-----------------------------------|-------|------|
|           |                           |                             |                             | Krill                             | Squid | Fish |
| Elephant  | 600                       | 0.50                        | 0.30                        | —                                 | 4.5   | 1.5  |
| Leopard   | 220                       | 0.27                        | 0.06                        | 0.5                               | 0.1   | 0.2  |
| Weddell   | 730                       | 0.25                        | 0.18                        | —                                 | 0.5   | 2.2  |
| Crabeater | 14,800                    | 0.19                        | 2.87                        | 63.2                              | 1.3   | 2.0  |
| Ross      | 220                       | 0.17                        | 0.04                        | 0.1                               | 0.6   | 0.2  |
| Fur       | 200                       | 0.05                        | 0.01                        | 0.1                               | 0.1   | 0.1  |
|           | 16,800                    | —                           | 3.46                        | 63.9                              | 7.1   | 6.2  |

reduced competition from blue, fin and humpback, but it could equally reflect some slow environmental change.

There is clear-cut evidence that pregnancy rates for blue, fin and sei whales have risen, and that the mean ages at sexual maturity have fallen, since intensive whaling was resumed after World War II. For the fin, the age at maturity was steady around 10 years from 1910–30, and has declined to 5–6 years in the most recent age classes. This suggests, again indirectly, increased food abundance. Similar evidence has been adduced for the crab-eater seal. In the Antarctic Peninsular, the mean age at sexual maturity for this krill-eater was 4 years until the whaling zone known as the 'Sanctuary'

was reopened; since then, the age has fallen to 2.5 years.

The question of the rate at which minke whale stocks are increasing, presumably in response to the effective disappearance of its close competitor the blue (see Fig. 1), is currently the subject of some dispute (IWC, *Rep. int. Whal. Commn.* **28**, 55; 1978).

Laws (II) concludes, "the baleen whales probably remain the most important vertebrate group in their effect on the antarctic ecosystem. Their stocks are likely to stabilise or slowly increase with realistic scientific management, but it is uncertain whether their original abundance will be regained even if commercial whaling ceases". □

## X-ray absorption spectroscopy

from C. David Garner

ALTHOUGH the existence of fine structure on X-ray absorption edges has been well known for more than 40 years, it is only recently that such data have been shown to provide reliable information concerning the electronic structure and the atomic arrangement about specific atoms in simple or complex materials. This transformation has been made possible by exploitation of the intense continuum of electromagnetic radiation, extending to wavelengths below 1 Å, available from high-energy electron or positron accelerators as a natural consequence of maintaining the charged particles in a circular trajectory. This radiation has a number of unique properties (Rowe & Weaver *Sci. Amer.* **236**, 32; 1977) and is generally known as synchrotron radiation.

The structural information derives from an accurate measurement of the variations in the X-ray absorption coefficient at and above absorption edge(s) of the element(s) of interest (Sayers, Lytle & Stern, *Adv. X-Ray Anal.*, **13**, 248; 1970; Stern *Sci. Amer.* **234**, 96; 1976). Present monochromator design and other limitations effectively restrict studies to the *K*-edges of the elements Ca–Mo and the *L*-edges of elements Ag–U. It is convenient to discuss the potential information contained within the X-ray absorption spectrum as three topics. (1) The actual energy of a particular absorption edge of an element depends on the oxidation state and the nature of the immediate chemical environment of that element. Typically, one unit increase of oxidation state increases the position of the edge by 1–3 eV. (2) The excitation of

the core electron into the continuum may be incorporated with transitions from the inner core level to outer bound levels. These internal excitations are usually evident as discontinuities on the primary absorption edge (edge structure) and they can provide information about the electron structure at the absorbing atom in the material of interest. (3) The emitted photoelectron can be considered as a spherical wave radiating from the absorbing atom. This wave will be partially reflected by the electron clouds of the atoms surrounding the absorber; the resultant interference between outgoing and back-reflected waves is manifest as a modulation of the X-ray absorption coefficient at energies greater than that of the absorption edge. This extended fine structure (EXAFS) thus contains information about the number and the type of atoms which surround the absorber and the corresponding interatomic distances. Many of the theoretical problems associated with the interpretation of EXAFS data now seem to have been overcome (Lee & Pendry *Phys. Rev. B* **11**, 2795; 1975; Teo, Lee *et al.*, *J. Amer. Chem. Soc.* **99**, 3854; 1977) and the validity of the structural predictions have been confirmed in a number of studies on materials of a known structure. (Hodgson *et al. J. Amer. Chem. Soc.* **100**, 2748; 1978).

X-ray absorption spectroscopy offers two particular advantages for the study of metal ions in biological systems. First, there is no requirement that the material of interest be crystalline, in principle gases, liquids, glasses and amorphous solids can be investigated. Second, the measurements are specific to the element of interest which may be present at a low concentration, say

<1 atom in 10<sup>4</sup>. Several investigations of the coordination about a metal ion in a metalloprotein have now been accomplished and the precision of the bond length data, which typically extended to ~3 Å from the primary absorber, can be ±0.02 Å. A unique and valuable insight into the nature of the molybdenum centre of the nitrogenases, has been provided by the measurement and interpretation of EXAFS data for the Mo–Fe protein of *A. vinelandii* and *C. pasteurianum* and the FeMo cofactor of the former (Hodgson *et al. J. Amer. Chem. Soc.* **100**, 3398, 3814; 1978). EXAFS studies for *P. aerogenes* rubredoxin (Shulman *et al. Proc. natn. Acad. Sci. U.S.A.* **72**, 4003; 1975) indicated that all four Fe–S bonds were of equal length, in contrast to the initial prediction of three long and one short Fe–S bond from an X-ray crystallographic structure determination. Subsequent revision of the latter led to a concordance between the two approaches. A similar unanimity exists between the EXAFS and X-ray diffraction studies accomplished for the copper centre of the 'blue' proteins (Hodgson *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 4069; 1978; Freeman *et al. Nature*, **272**, 319; 1978).

However, the EXAFS determinations of the Fe–N distances in oxy and deoxyhaemoglobin (Eisenberger *et al. Proc. natn. Acad. Sci. U.S.A.* **73**, 491; 1976) have indicated that the iron atom moves much less than the 0.7 Å away from the haem plane suggested from the Hoard–Perutz model. It is thus suggested that the cooperativity between the haem centres does not originate from a significant displacement of the iron atom upon oxygenation.

The paper by Labhardt and Yuen in this issue of *Nature* (page 154) describes the results of an X-ray absorption spectroscopic study of the iron centre of cytochrome *c*. In contrast to the results of an earlier study (Shulman *et al. Proc. natn. Acad. Sci. U.S.A.* **73**, 1384; 1976), a shift in the iron *K*-edge position and the association edge structure of ~1.5 eV was observed on oxidation. The sense and magnitude of the shift are consistent with a redox process which is localised at the iron centre. Furthermore, the EXAFS details indicate that the lengths of bonds involving the iron atom do not change on oxidation (although there is no indication in this paper that these details are consistent with those expected from the studies of Dickerson *et al. J. biol. Chem.* **248**, 5234; 1973). Therefore, the two principal conclusions from this study are compatible with a view that, as expected from Frank–Condon considerations, electron transfer metallo-



proteins contain centres for which the redox change, although primarily located on the metal centre, does not require nor lead to a change in the geometry of the immediate environment of the metal. The EXAFS results for cytochrome *c* and haemoglobin indicate that the conformational changes in the peptide, which register the change in state of the metal centre, almost certainly do not originate as a result of changes in the iron-ligand bond lengths. Labhardt and Yuen suggest that the conformational transition may be initiated by a change in the amplitude of certain low frequency vibrations. This is possible, although, in contrast to their specifications, it seems more likely that the reduced iron centre would lead to a more flexible structure than its oxidised counterpart. Thus the weaker metal-ligand interactions expected for the reduced centre would be expected to result in vibrational amplitudes, for example, for

metal-ligand bending modes which, although corresponding to approximately the same average interatomic distances, would be larger than obtained for the corresponding oxidised system at the same temperature.

A real assessment of the contribution of X-ray absorption spectroscopy must await a wider and more detailed series of studies than accomplished thus far. Certain of the present limitations which restrict the range of systems which may be studied and potential interpretive ambiguities will undoubtedly be reduced in the near future. However, there seems to be no prospect of obtaining angular as well as radial structural data. Nevertheless, there is no doubt that the validity of this technique is well established and that it offers unique opportunities for the detailed investigations in a wide range of systems. The impact on inorganic biochemistry should be particularly significant. □

## Coated vesicles

from M. Geisow

GLUTS or shortages of the membranes which enclose cells and their constituent organelles arise under some circumstances. For example, during endocytosis, the plasma membrane of cells is progressively depleted as the cytoplasm becomes filled with endocytotic vesicles. Conversely, following the stimulated release of cell products, excess membrane accumulates at the cell surface at the expense of intracellular pools. In both cases a recovery system is necessary. Reversion to a resting state is achieved in many exocytotic systems by the interiorisation of excess plasma membrane. Vesicles return through the cytoplasm either to subcellular membranes or to lysosomes where they are degraded. Whilst little is known about the nature and composition of such vesicles, recent progress has been made with one class of vesicles with a curious polyhedral 'exoskeleton' which encloses their membranes. This structure has given them the name 'coated vesicles'.

Coated vesicles have been recognised for many years. They were first observed in association with embryonic nutrition, where invaginations of the oocyte membranes were seen to take up food from the external environment into vesicles with a 'bristle coat'. Subsequently many workers have noted this feature in both endocytotic and exocytotic profiles. Palade and his colleagues reported coated vesicles in the Golgi region of secreting cells. Franke *et al.* (*J. Cell Biol.* **69**, 173;

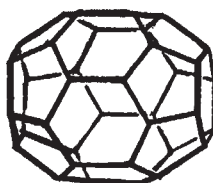


Fig. 1 One structure assigned by Crowther *et al.* to vesicle coats. 1976) identified an exported product of mammary gland (casein) in coated secretory vesicles.

Later studies have suggested that the coated vesicle might represent a device for recycling cell membrane. In frog neuromuscular junction Heuser and Reese (*J. Cell Biol.* **57**, 315; 1978) described the sequential fusion of synaptic vesicles with junctional membrane followed by the pinocytosis of presynaptic membrane as coated pits which became coated vesicles. The earlier work of Holtzman *et al.* (*Science* **173**, 733; 1971) with lobster neuromuscular junctions was supported by these findings, although the latter article reported few coated vesicles.

Barbara Pearse has devised a procedure for isolating coated vesicles from various tissues (*J. molec. Biol.* **97**, 93; 1975). Their lattice-like coats are composed predominantly of one type of 180,000 molecular weight polypeptide which she has called clathrin. Several analyses of the coat morphology have been made (for example Kaneski & Kadoki *J. Cell Biol.* **42**,

202; 1969). This and more recent studies (Crowther *et al.* *J. molec. Biol.* **103**, 785; 1976; Woods *et al.* *J. Cell Sci.* **30**, 87; 1978) indicate that the clathrin lattice is formed from trimeric associations of clathrin to give hexagonal and pentagonal units (Fig. 1). Alteration in the number of hexagonal units allows the size of the enclosed vesicles to vary without distortion of the lattice.

Peptide maps of clathrin prepared from different species indicate that the protein is strongly conserved (Pearse *Proc. natn. Acad. Sci. U.S.A.* **73**, 1255; 1976). There are also close similarities in the more minor components of coated vesicles isolated from different tissues (Pearse *J. molec. Biol.* **126**, 803; 1978). Pearse suggests that some of these might have a structural role, perhaps relating to the vertices of the polyhedral coat. However, the number of polypeptides obtained from the isolated coated vesicles is small, which is surprising in view of the ultrastructural evidence that they participate in endocytotic and exocytotic transport. Both Pearse and also Blitz *et al.* (*J. Cell Biol.* **75**, 135; 1977) report polypeptides of 100,000 and 55,000 molecular weight. The latter workers consider that these molecules resemble the major constituents of sarcoplasmic reticulum. They therefore infer that the molecules belong to the transported vesicle rather than to its polyhedral coat. The larger polypeptide in particular appears to be a calcium-transporting ATPase and cross reacts with antisera to the authentic sarcoplasmic reticulum enzyme. It is notable that intracellular and pericellular membrane pools for which the coated vesicle may act as a go-between, contain substantial amounts of calcium-stimulated ATPase (for example, Golgi membranes: Hodson *J. Cell Biol.* **30**, 117; 1978), endoplasmic reticulum: Macdonald *et al.* *J. biol. Chem.* **253**, 3504; 1978). More work is needed, however, to establish firmly the nature of the components in coated vesicles.

Other intrinsic membrane proteins may be carried by the enclosed vesicle. For example, Gorden *et al.* (*Proc. natn. Acad. Sci. U.S.A.* **75**, 5025; 1978) in a study of the binding of <sup>125</sup>I-epidermal growth factor (EGF) to human fibroblasts found that one-third of the specifically bound label rapidly became associated with bristle-coated areas of the plasma membrane (representing less than 2% of the total cell surface). Label and membrane were pinocytosed to form a coated vesicle. It seems plausible that the EGF receptor was also lost in this way, as prolonged stimulation depleted the surface receptors.

Many questions remain unanswered about coated vesicles in particular and

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membrane transport in general. Understanding the control of the assembly, disassembly and destination of coated vesicles now provides an experimental challenge—but why is the cage structure necessary at all? One suggestion has been made that the clathrin physically pinches off a membrane vesicle and at the same time controls the size of the final structure. Another suggestion might be made here. It is widely held that cellular membranes retain their identity with respect to membrane components, even when extensive membrane transport occurs. Is it possible that the closely-applied clathrin molecules provide a 'filter' for some categories of membrane protein, preventing their equilibration between pericellular and subcellular pools? □

## Prospects for solar energy

from Elizabeth Michelson

THE construction and monitoring of both highly-insulated experimental solar heated houses and solar installations in conventional housing is becoming quite widespread. At a recent meeting held in London\* G. O. G. Löf reported on experimental houses that have been built at Colorado State University to compare different types of solar heating systems. Air-circulating systems, with rock storage, were found to supply much the same amount of energy as flat-plate water collectors but were preferable on grounds of cost, durability and lack of problems of corrosion or damage from leakage. In the UK the Building Research Establishment at Watford is to install identical solar heating systems in 80 existing typical 3-bedroomed houses, in order to give a distribution of total daily hot water use, S. J. Leach reported. The ratio of panel area to total daily hot water use and the hot water delivery temperature are the main parameters determining the system performance (B. J. Brinkworth, University College, Cardiff).

There were many shades of opinion on the economic feasibility of solar domestic heating, largely because it rests on speculation as to fuel costs and the general economic situation in the near future. Leach held that a system could only be regarded as economic if it were so now but Brinkworth pointed out that insufficient work has yet been done on the durability of systems, and that this has to be taken into account if the economic viability is to be determined in any

set of circumstances. In general, it was agreed, solar water heating is certainly practicable in the UK.

H. Hörster (Philips, Aachen) concluded from theoretical studies that space heating was uneconomic at present in North European or UK conditions. An average house would cost some £2,500 to convert it to the high standard of insulation (as in the Swedish building regulations) needed before space heating is economically feasible. In the South of France however, solar space heating is a good prospect even in the normal house. Between sixty and seventy per cent of US homes already have air-circulating central heating systems and Löf considered that air collectors for space heating, which are presently less common, will become competitive in the near future.

### Solar ponds

A solar pond is a water-filled pond, 1-2 m deep, with a blackened bottom for greater heat absorption and a gradually increasing salt concentration towards the base to eliminate convection, which is the main cause of heat loss (H. Tabor, The Hebrew University, Jerusalem). The hot water remains at the bottom from which it is removed as required. Solar ponds tackle several of the major problems associated with other types of collectors; they can easily cover a large area and act as the store at the same time. In Israel, a solar pond will collect about 20% of the incident radiation and Tabor estimated that 200 km<sup>2</sup> of solar ponds would supply all of Israel's current demand for electricity. However, owing to the low ratio of winter to summer radiation, solar ponds are unsuited to high latitudes.

### Solar cells

Solar or photovoltaic cells convert light directly into electricity. Of these, single-crystal silicon cells are so far the only reliable and durable ones and have been used for many years in space and for the generation of small quantities of electricity in remote areas (H. Durand, Commissariat à l'Energie Solaire, Paris). Heterojunction cells, in which the semiconductor is made up of two different materials, have the advantage of requiring a much lower-temperature manufacturing process, but have not yet equalled the efficiency and durability of silicon cells (S. Wagner, Solar Energy Research Institute, Golden, Colorado). Optimism for the extensive use of solar cells in the future is witnessed by the passing of the US Photovoltaic Bill allocating \$1.5 billion over the next 10 years, \$125 million for the present year (Durand). The corresponding present

annual budget for Europe is £2.5 million for France and £2 million for West Germany. However, the attractive prospect of direct electricity has cost as one of its chief drawbacks. At present, 1 kW costs between £20,000 and £25,000; improved technology and mass production could reduce this by a factor of thirty. At the same time, the present efficiency of about 16% should not fall below 10% for the costs of encapsulation and installation to remain covered. The US hopes to add solar-generated electricity to its national grid, which can absorb random inputs of  $\pm 15\%$ , since large storage facilities are not feasible.

### Energy from biomass

The two most interesting end-products of the action of microorganisms on organic material are alcohol (ethanol) and methane (R. C. Righelato, University of Reading). The production of ethanol by fermentation is very costly because a dilute aqueous environment is required for the reaction and a large quantity of energy, comparable with the energy content of the ethanol, is required to recover the latter from solution. Improvements can be made by finding a method for recovering the alcohol which is less energy demanding than distillation, or using less water initially. Some yeasts can act in concentrations of up to 20% if only for a short time. Ethanol has been produced as a fuel in Brazil, though it is unlikely to make much contribution in the UK.

Gaseous products such as methane emerge from the solution unaided. The production of methane from sewage is more efficient than fermentation, requires less energy input and processes all the material including the cellulose. Methane production has already shown itself to be very practical and is particularly suited to small installations on farms.

As solar collectors, plants have some considerable advantages over man-made collectors. They have large surface areas, built-in storage and an enormous diversity, adapted to almost all conditions (N. K. Boardman, CSIRO, Dickson, Australia). However, the average efficiency of conversion of solar energy is only about 1%. The yield can be increased by the use of fertilisers, coppicing and short rotation crops, though the long-term environmental consequences of these are as yet unknown. Crop residues should also be utilised, but some must remain on the soil to inhibit wind and water erosion and supply nutrients. The crop can either be used as a dry fuel (such as timber) or converted to

\*A discussion meeting on Solar Energy was held on 15-16 November, 1978 at the Royal Society, London.

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ethanol, methane or oil with conversion efficiencies of 20–44% for gases and 58% for oil. Boardman estimated that such liquid fuels could contribute 47% of Australia's transport fuel requirements, one-third originating in crop residue and two-thirds in new fuel crops. Similarly good possibilities exist in Brazil and New Zealand. However, biomass is unlikely to make a significant contribution to the energy requirements of small densely populated countries.

The overall impression was one of optimism, based not on the all-embracing potential of any one method, but on the diversity of methods of harnessing solar energy which, given a rational energy consumption, can make some contribution in almost every climate. □

## From sea level to heat flow

from Peter J. Smith

WHEN hot material of mantle origin spreads away from an oceanic ridge zone it cools and contracts. Both theory and observation indicate that the seafloor subsidence thereby produced is proportional to the square root of time, from which it follows that a fast-spreading ridge will have a greater volume than a slow-spreading one. It follows from that, in turn, that, assuming the volume of seawater and the radius of the Earth to remain constant, the sea level will rise during a period of fast spreading. So could variations in ridge spreading rate be responsible for some or all of the fluctuations in sea level that have been known now for more than a hundred years? Hays and Pitman (*Nature* **246**, 18; 1973) thought so and thus added to the large number of explanations previously suggested for changing sea level (glaciation, continental volume changes, Earth's radius changes).

Now Turcotte and Burke (*Earth Planet. Sci. Lett.* **41**, 341; 1978) have taken the logic of this model one stage further and, assuming sea level fluctuations indeed to be indicative of changes in ridge volume, have related changes in sea level to changes in ridge heat flow. The sea level data used were obtained indirectly from Hallam (*Nature* **269**, 769; 1977), who estimated the percentage flooding of North America and the Soviet Union over the past 520 million years from palaeogeographic maps, and from Vail *et al.*

(*Am. Assoc. Pet. Geol. Mem.* **26**, 83; 1978), who determined relative sea levels directly for the past 600 million years using a combination of seismology and stratigraphy to detect the onlap of coastal deposits in maritime sequences. The two data sets suggested remarkably similar sea level trends, thereby giving some confidence in the apparent changes in water volume, and hence the supposed changes in ridge volume derived from them, taking into account isostatic responses.

Relating ridge volume to ridge heat flow is a more complex operation in the mathematical sense but can be done using thermal boundary layer theory. Expressing the ridge heat flow as a proportion of total world heat flow is rather simpler, however, although the number of assumptions required (for example, that the Earth's heat is entirely due to uranium, thorium and potassium in the Earth) begins to mount alarmingly. Given the number of assumptions involved and the considerable uncertainty in many of the numerical values used, Turcotte and Burke are cautious in interpretation. Nevertheless they feel justified in coming to two tentative general conclusions—first, that the proportion of the Earth's heat dissipation occurring in ridge zones has generally declined slowly over the past 600 million years much in accord with the decline in heat generation over that period, and, second, that there have been times during the Phanerozoic when the proportion of the Earth's heat dissipation occurring in ridge zones, at present 28 per cent, has risen as high as 50 per cent.

According to the interlocking model thus far developed, these ridge heat flow 'highs' coincide, of course, with sea level maxima and hence with the times of the most extensive continental flooding—and the latter occurred about 80 and 400–500 million years ago. The 600 million years of observation is far too short to support any suggestion that these figures indicate cyclic behaviour with a periodicity of about 300 million years, although it is interesting to note that 300 million years is about equal to the turnover time of a mantle convection cell. Moreover, if sea level and ridge heat flow maxima are indeed causally related, both would be expected to be related to convective behaviour, for heat flow variations almost certainly are.

And that leads to another thought. High ridge heat flows correspond with ridge volume maxima which coincide with fast ridge spreading. In turn, this must coincide with increases in the amount of mantle material upwelling at ridges which (assuming an Earth of constant radius) must be connected

with increases in the rate of lithospheric consumption at subduction zones. As orogenic behaviour is mainly associated with plate consumption, the implication here is that orogenic processes are also episodic, reaching greatest intensity when the sea level is highest. If it be true, as Turcotte and Burke argue, that the widespread destruction of rocks and structures during mountain building obscures much of the evidence of how and when mountains formed, sea level studies may turn out to be useful in determining the precise temporal variations in orogenic activity. □



## A hundred years ago

I HAVE just read in *Nature* (vol. xix, p. 182) an account of a strange electrical phenomenon observed at Teignmouth. In connection with it the following incident may be of some interest:—When in Switzerland, not long since, I made with some friends the ascent of Monte Rosa. The weather was unsettled, and on gaining the summit we saw a thunderstorm advancing in our direction from the Italian valleys, and not wishing to turn ourselves into lightning-conductors we deemed it wise to retire from the summit. We had retreated a very short distance along the *arête* when the storm-clouds swept up upon us; the fine snow fell so thick that we could hardly see one another, and we were all suddenly attracted by a peculiar ticking or fizzing from our hair; when I held up my axe the ticking was most distinctly heard from the top of it. The thunder ceased, and we felt that we were acting as points, through which the ground electricity was flowing off into the cloud; if it had been dark, the bluish light observed at Teignmouth might have been visible.

As at Teignmouth, so on Monte Rosa; it was freezing hard when the phenomenon was observed.

W. S. GREEN

Alta Terrace

Monkstown, Cork

From *Nature* **19**, 9 Jan., 220; 1879.

## Erratum

In the article 'Discrepancies in claims for endothermy in therapsids and dinosaurs' (*News and Views* **276**, 757; 1978) the last eight lines of paragraph 1 should read: "As usual some disputed issues reflect different individual views of identical evidence, but one of Bakker's later arguments involves a serious factual error, unless many other authors are mistaken, while some of the arguments used in different papers are inconsistent or contradictory."

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# review article

## Multiple receptors for dopamine

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*Pharmacological and biochemical criteria can be used to separate those dopamine receptors which are linked to the enzyme adenylyl cyclase and those which are not.*

BEFORE discussing the existence of multiple receptor mechanisms for dopamine, some general comments about the definition of receptors are appropriate. The concept of a 'receptor' for a substance arises from the ability of tissue both to recognise and respond to minute quantities of a substance in some precise way. Receptors may be defined according to many different criteria, but it is most profitable to identify the endogenous physiological agent which normally interacts with the receptor. The next stage of classification is based on a combination of pharmacology (that is, identification of drugs which selectively mimic or antagonise the physiological agent) with biochemistry, anatomy and physiology. This second level of categorisation is most useful when it generates the least number of subgroups; however, within a given category, further division is possible into a hierarchy of descending levels (for example, nicotinic cholinergic receptors are further subdivided into ganglionic and neuromuscular receptors). There are many precedents for the existence of multiple classes of receptors for a given biological molecule. Thus, acetylcholine interacts with nicotinic and muscarinic receptors, histamine with  $H_1$  and  $H_2$  receptors, and catecholamines with  $\alpha$ - and  $\beta$ -adrenergic receptors.

Dopamine receptors can be characterised by various criteria. In earlier discussions, dopaminergic receptors were classified on the basis of physiological<sup>1</sup> or pharmacological and biochemical<sup>2,3</sup> criteria; alternatively a dynamic equilibrium of a single dopamine receptor fluctuating between two states or configurations has been postulated<sup>4</sup>. The demonstration of a dopamine-sensitive adenylyl cyclase not only provided an *in vitro* model to study the properties of a dopamine receptor but also suggested a mechanism for the generation of the physiological response to this amine<sup>5</sup>. However, not all dopamine receptors have properties similar to those of the receptor linked to adenylyl cyclase. Furthermore, in some tissues, a dopamine-stimulated accumulation of cyclic AMP does not seem to be involved in the generation of the physiological response to dopamine.

### Dopamine receptors which increase cyclic AMP synthesis

One physiological action of dopamine is to increase the synthesis of cyclic AMP by the stimulation of a specific dopamine-sensi-

tive adenylyl cyclase<sup>6,7</sup> (for review see ref. 8). The dopamine-sensitive adenylyl cyclase is widely distributed, particularly in neural tissue ranging from gastropods<sup>9</sup> and insects<sup>10</sup> to human brain<sup>11</sup>. The demonstration of a dopamine-sensitive adenylyl cyclase in cell-free preparations of caudate nucleus<sup>6</sup> raised the possibility that the postsynaptic dopamine receptor in this region of the brain could regulate the enzyme. This concept provided the impetus for a detailed characterisation of the dopamine receptor associated with the striatal enzyme; the procedures used to define this receptor involved the use of dopamine itself, analogues of dopamine, and synthetic drugs which stimulate or block dopamine receptors.

Although many structural analogues of dopamine have been tested for their effects upon the striatal adenylyl cyclase, no catecholamine is more potent than dopamine itself. The only catechol-containing molecule which is equipotent with dopamine is epinine (*N*-methyldopamine); any other substitutions on the dopamine molecule produce a loss of agonist activity<sup>12-14</sup>. Summaries of these structure-activity studies have been published previously<sup>8,15</sup>. A number of analogues, which possess dopamine-like activity *in vivo*, have also been shown to mimic the ability of dopamine to increase adenylyl cyclase activity. The semirigid dopamine analogue, 6,7 dihydroxy-1,2,3,4-tetrahydronaphthalene (A-6, 7-DTN), is equipotent with dopamine as an *in vitro* agonist<sup>12</sup>. Other structural analogues give less consistent results. Apomorphine is 'classically' recognised to mimic the effects of dopamine<sup>16</sup>, but when tested on the striatal enzyme system, this molecule is only a partial agonist<sup>6</sup>; it can antagonise dopamine receptors both in cell-free homogenates<sup>6</sup> and in preparations of intact cells<sup>17</sup>.

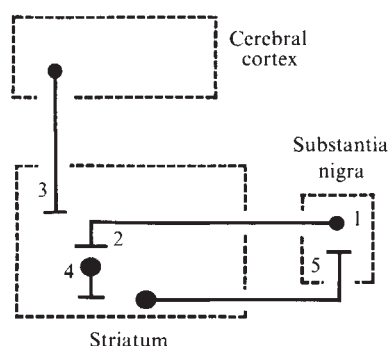
Antagonists have also been used to define the properties of the dopamine receptor which regulates striatal adenylyl cyclase. Classic antagonists of  $\alpha$ - and  $\beta$ -adrenergic receptors are relatively ineffective as antagonists of dopamine<sup>6</sup>, but antipsychotic drugs are potent, competitive antagonists<sup>6,11,18,19</sup>. Several dopamine antagonists exist as optical or stereoisomers; the stereoisomers of butaclamol and *cis*- and *trans*-flupenthixol are examples. The clinically active compounds (+)butaclamol and *cis*-flupenthixol are potent dopamine antagonists while the clinically and pharmacologically inactive compounds (-)butaclamol and *trans*-flupenthixol are less potent as dopamine antagonists<sup>18,20,21</sup>.



The relative imprecision of the experimental measures of *in vivo* dopaminergic function (for example, turning and stereotypic behaviour in rats, neurological or psychiatric changes in man) preclude an exact definition of the role of dopamine-sensitive adenylyl cyclase in the brain; although dopamine and cyclic AMP have been implicated in electrophysiological events in mammalian superior cervical ganglia<sup>22,23</sup> and in a *Helix* ganglion<sup>9</sup>. However, activation of dopamine receptors of isolated cells of the bovine parathyroid is followed by the accumulation of cyclic AMP and the release of parathyroid hormone<sup>17</sup>. This indicates that tissue-specific responses to dopamine can be mediated by the dopamine-sensitive adenylyl cyclase.

## Dopamine receptors which do not increase cyclic AMP synthesis

Other physiological responses to dopamine seem not to involve either the stimulation of an adenylyl cyclase or the accumulation of intracellular cyclic AMP. An example is the dopamine-induced inhibition of prolactin release from mammothrophs.



**Fig. 1** A schematic representation of the neurones in the nigro-striatal axis which contain dopamine receptors. The dopaminergic neurones contain autoreceptors which regulate either electrical firing of these cells in the substantia nigra (site 1) or tyrosine hydroxylase activity (site 2) on the terminals. Cortical neurones which project to the striatum possess binding sites for radiolabelled haloperidol which have been identified as dopamine receptors (site 3). Neurones intrinsic to the caudate contain the dopamine-sensitive adenylyl cyclase activity (site 4). Finally, terminals of striatal neurones which project to the nigra also contain a dopamine-sensitive adenylyl cyclase (site 5). The dopamine receptors at sites 1–3 have not been associated with adenylyl cyclase activity; only the receptors at sites 4 and 5 regulate this enzyme activity (for references see text).

The synthesis and release of prolactin are under a predominantly inhibitory hypothalamic influence; dopamine in the median eminence functions as an endogenous hypothalamic factor which inhibits prolactin release (for comprehensive reviews see refs 24, 25). The anatomical, physiological, pharmacological and biochemical evidence that demonstrates the presence of receptors for dopamine on the mammothrophs includes the following evidence: (1) Catecholamines, including dopamine, can inhibit the release of prolactin *in vivo* and *in vitro*<sup>26,27</sup>. (2) Dopaminergic agonists, such as apomorphine can mimic the inhibitory effect of dopamine. (3) The effects of dopamine can be blocked by several selective dopaminergic antagonists<sup>28,30</sup>. (4) Radiolabelled agonists and antagonists of this receptor bind with high affinity to stereospecific sites that have properties similar to those of the dopamine receptor on the mammothrophs<sup>30–32</sup>.

There is no evidence that a dopamine-stimulated accumulation of cyclic AMP can account for the inhibitory effect of

dopamine on prolactin release. Cholera toxin, the nonspecific activator of adenylyl cyclase (see ref. 33 for review) has been added to cells of the anterior pituitary or cloned tumour cells derived from the anterior pituitary. Cholera toxin either stimulates<sup>34</sup> or has no effect on the release and synthesis of prolactin (M. Thorner, personal communication); it never decreases prolactin release. Furthermore, dopamine inhibits the adenylyl cyclase from prolactin secreting human pituitary adenomas<sup>35</sup>.

A second example of a dopamine receptor which does not stimulate adenylyl cyclase activity is the dopamine receptor (autoreceptor) on the dopaminergic nigro-neostriatal neurones, which are found on presynaptic terminals in the striatum and also on the cell bodies of the same neurones in the substantia nigra<sup>36,37</sup>. Although a dopamine-sensitive adenylyl cyclase has been identified in the substantia nigra<sup>38,39</sup> as well as the striatum<sup>6</sup>, the enzyme occurs on cellular elements other than the dopaminergic neurones. Lesions in the nigra induced by 6-hydroxydopamine (which selectively destroys the dopaminergic neurones) do not alter the activity of dopamine-sensitive adenylyl cyclase within either the nigra<sup>39</sup> or the striatum<sup>40</sup>. Conversely, intrastriatal injection of kainic acid, a rigid glutamic acid analogue, destroys neuronal cell bodies in the striatum but spares the dopaminergic nerve terminals; this procedure causes a substantial loss of striatal dopamine-sensitive adenylyl cyclase activity but does not diminish the content of dopamine<sup>41,42</sup>. Thus neither the autoreceptors upon cell bodies in the nigra nor the autoreceptors on the terminals in the striatum appear to be associated with a dopamine-sensitive adenylyl cyclase.

## Pharmacological separation of dopamine receptors

Pharmacological evidence indicates the existence of several classes of dopamine receptors. Several antagonists are not equally effective upon different dopamine receptors. For example, the antipsychotic drugs, molindone and sulpiride, and the antiemetic drug, metoclopramide, are dopamine antagonists when tested in the anterior pituitary<sup>43–45</sup> or the brain<sup>47,75</sup>. However, neither metoclopramide nor sulpiride blocks striatal dopamine-sensitive adenylyl cyclase<sup>3,46,75</sup>. Molindone is, at most, a weak antagonist of this enzyme (B. K. Krueger and P. Greengard, personal communication; M. Munemura and J. W. Kebabian, unpublished observation).

Other compounds are capable of a more limited discrimination between different dopamine receptors. Dopamine itself displays a considerable range of potency at its receptors in different tissues. Nanomolar concentrations of dopamine elicit a response from either the anterior pituitary ( $K_a = 35$  nM) or the presynaptic dopaminergic receptors on the sympathetic terminals in the artery of the rabbit ear ( $K_a = 37$  nM)<sup>30,48</sup>. The presynaptic autoreceptor on the terminals of the nigro-neostriatal neurones is stimulated by somewhat higher concentrations of dopamine<sup>49</sup>. In contrast, dopamine receptors linked to adenylyl cyclase are stimulated by micromolar concentrations of dopamine. This observation has been made repeatedly in cell-free homogenates of neural tissue derived from species as divergent as the cockroach ( $K_a = 2$   $\mu$ M)<sup>10</sup> and man ( $K_a = 6$   $\mu$ M). Micromolar concentrations of dopamine are also necessary for the accumulation of cyclic AMP in preparations of tissue containing intact cells<sup>5</sup>. Finally, micromolar concentrations of dopamine are required either to stimulate cyclic AMP accumulation or to elicit release of parathyroid hormone in preparations of the bovine parathyroid<sup>17</sup>. Thus, the relative potency of dopamine can be the basis of a categorisation of dopamine receptors.

Apomorphine is another compound with selective activity on different dopamine receptors. In many tissues, apomorphine mimics the effects of dopamine; both dopamine ( $K_a = 35$  nM) and apomorphine ( $K_a = 3$  nM) are potent activators of the pituitary dopamine receptor<sup>30</sup>. Apomorphine ( $K_a = 44$  nM) also

mimics the effect of dopamine ( $K_a = 37$  nM) on the sympathetic presynaptic receptors of the rabbit ear artery<sup>48</sup>; the potency of apomorphine ( $K_a = 300$  nM) and dopamine ( $K_a = 320$  nM) on the autoreceptor on the terminals of the nigro-neostriatal neurones are similar<sup>49</sup>. In contrast, in several tissues where dopamine receptors are linked to the enzyme adenylyl cyclase, apomorphine displays activity as a dopamine antagonist. In the striatal dopamine-sensitive adenylyl cyclase system, apomorphine has actions as both an agonist (at low concentrations) and an antagonist (at higher concentrations)<sup>6</sup>; similar observations have been made in kidney vasculature<sup>50</sup>. Apomorphine antagonises the dopamine-stimulated accumulation of cyclic AMP in the bovine parathyroid; the compound has no agonist activity on these cells<sup>17</sup>. Thus, the different pharmacological actions of apomorphine imply the occurrence of more than one type of receptor.

Several ergot derivatives can also distinguish different dopamine receptors. The dopaminergic ergots, such as bromocriptine, lisuride and lergotril, are extremely potent agonists of the dopamine receptor of the mammothrophs. When tested on

Although antipsychotic agents are potent antagonists of dopamine receptors in many tissues, these drugs are of only limited use in discriminating between the different types of dopamine receptors. Various chemically distinct antipsychotic drugs are antagonists of the dopamine receptor on mammothrophs<sup>24</sup>; the potency of some of these compounds has been estimated *in vitro*<sup>30</sup>. In addition, antipsychotic drugs are powerful antagonists of the presynaptic dopamine receptors of the rabbit sympathetic neurones<sup>48</sup> and the autoreceptors on the dopamine-containing nigro-neostriatal neurones<sup>49</sup>. Antipsychotic drugs are also antagonists of the dopamine receptor which is linked to striatal adenylyl cyclase<sup>6,11,18,19</sup>.

Among the different classes of antipsychotic compounds, only the butyrophenone series contributes to the identification of distinct types of receptors for dopamine. Butyrophenones, such as haloperidol, are less potent antagonists of the dopamine receptor linked to adenylyl cyclase than would be anticipated from their potency as antipsychotic agents<sup>57</sup> or antagonists of the dopamine receptor in the anterior pituitary<sup>30</sup>. Other antipsychotic compounds do not display such differential activity. For example, the dopamine receptors in the anterior pituitary and those linked to striatal adenylyl cyclase display approximately equivalent affinities for members of the thioxanthine series (for example, *cis*-flupenthixol), the thiothixene series (for example, *cis*-thiothixene) and (+)butaclamol<sup>6,11,18,19,30</sup>.

**Table 1** Criteria for the classification of dopamine receptors

| Name*                           | D-1                                                                       | D-2                               |
|---------------------------------|---------------------------------------------------------------------------|-----------------------------------|
| Cyclase linkage                 | Yes                                                                       | No                                |
| Location of proto-type receptor | Bovine parathyroid                                                        | Mammothroph of anterior pituitary |
| Dopamine                        | Agonist ( $\mu$ molar potency)                                            | Agonist (nmolar potency)          |
| Apomorphine                     | Partial agonist or antagonist                                             | Agonist (nmolar potency)          |
| Dopaminergic ergots             | Potent antagonist (nmolar potency)<br>Weak agonist ( $\mu$ molar potency) | Agonist (nmolar potency)          |
| Selective antagonist            | None known as yet                                                         | Metoclopramide<br>sulpiride       |
| Radiolabelled ligand            | <i>cis</i> -flupenthixol†                                                 | Dihydroergocryptine               |

\* Previously<sup>65</sup>, we have designated the two categories of dopamine receptors as ' $\alpha$ -dopaminergic' and ' $\beta$ -dopaminergic'. This has led to confusion with the  $\alpha$  and  $\beta$  adrenoceptors. The new designations should prevent further confusion.

† Radiolabelled *cis*-flupenthixol can be used as a ligand specific for the dopamine receptor linked to adenylyl cyclase in the rat striatum<sup>64</sup>. Its affinity for the dopamine receptor in the anterior pituitary has not been measured.

isolated cells of the anterior pituitary, bromocriptine has a  $K_a$  of 2.9 nM, which is approximately 10-fold more potent than dopamine itself ( $K_a = 35$  nM)<sup>30</sup>. Extremely high concentrations of the ergots can cause accumulation of cyclic AMP in some tissues, thereby mimicking the action of dopamine. For example, bromocriptine, like dopamine, causes an accumulation of cyclic AMP in the rabbit retina, but this effect requires a 33,000-fold higher concentration than is needed to inhibit prolactin release<sup>51</sup>. Of greater interest is the ability of such ergots to block the striatal dopamine receptor linked to adenylyl cyclase. This antagonist activity, which is unanticipated in view of the action of such compounds on the anterior pituitary, is seen in both intact and homogenised preparations of striatal tissue<sup>52,53</sup>. When tested in the cell-free striatal homogenate, two dopaminergic ergots, lisuride<sup>54</sup> and lergotril<sup>55,56</sup> are devoid of agonist activity; both of these compounds are antagonists of the dopamine receptor. These differences in potency and action of the dopaminergic ergots point to differences between the dopamine receptors found in the pituitary and dopamine receptors associated with adenylyl cyclase in the striatum.

## Radiolabelled ligands

A number of dopaminergic drugs, radiolabelled to high specific activity, have been used to identify specific neuronal binding sites which may correspond to dopamine receptors. Impressive correlations have been established between the affinity of certain dopaminergic drugs for these specific binding sites and their potency in animal and human tests of central dopaminergic neurotransmission<sup>58</sup>. The physiological and biochemical function of the central dopamine receptors identified by binding studies are only just beginning to be understood.

The dopamine receptors in the anterior pituitary have been studied with three different radiolabelled ligands, spiroperidol<sup>32</sup>, dihydroergocryptine<sup>30</sup> and dopamine<sup>31</sup>. Because the properties of this receptor are well characterised, a correlation between the binding sites and the receptor has been possible<sup>30,31</sup>. There is no direct evidence to suggest that any of the binding sites identified in the striatum with radiolabelled dopamine<sup>58</sup>, haloperidol<sup>4,59</sup>, spiroperidol<sup>32</sup>, apomorphine<sup>60</sup>, dihydroergocryptine<sup>61</sup> or ADTN<sup>62</sup> are directly related to the dopamine receptor which regulates adenylyl cyclase activity in this region. Recent studies with kainic acid lesions and ablations of the cerebral cortex provide further evidence to distinguish some of the specific binding sites from the dopamine-sensitive adenylyl cyclase in the striatum<sup>63</sup>. These experiments imply that the striatal dopamine-sensitive adenylyl cyclase is located predominantly on neurones intrinsic to the caudate; in contrast, most of the striatal haloperidol binding sites are located on nerve terminals derived from neurones in the cerebral cortex. Recently, specific binding sites with properties similar to the dopamine receptor regulating striatal adenylyl cyclase have been identified in experiments with radiolabelled *cis*-flupenthixol<sup>64</sup>.

## A classification of dopamine receptors

It is evident that dopamine receptors can be divided by biochemical or pharmacological criteria. The bovine parathyroid provides an example of a type of dopamine receptor which stimulates the enzyme adenylyl cyclase, whereas the mammothrophs of the anterior pituitary contain dopamine receptors which do not stimulate adenylyl cyclase. We propose that the dopamine receptors in these two tissues are examples of two distinct categories of receptor. The properties of these two types of dopamine receptors, which we designate D-1 and D-2, are summarised in Table 1. This is only the first level of division in



the classification of dopamine receptors; it is likely that with further examination, particularly as pharmacological evidence accumulates, subcategories of each type of receptor will be discerned. For example, the autoreceptors on terminals of the nigro-neostriatal neurones can be differentiated from the dopamine receptors in the anterior pituitary<sup>66</sup>.

## Discussion

As an example of the location of different dopamine receptors within a tissue, it is of interest to examine the striatum, the most extensively studied dopaminergic brain region. We are able to identify dopamine receptors at five sites within the nigro-striatal axis (Fig. 1). The dopamine receptors at three of these sites are not associated with adenylyl cyclase (and therefore would be designated D-2 dopamine receptors in our classification); at two of the sites, the dopamine receptors are linked to adenylyl cyclase (and therefore would be designated D-1 dopamine receptors). In the striatum there are: (1) presynaptic receptors which control tyrosine hydroxylase and are unassociated with an adenylyl cyclase; (2) postsynaptic receptors responding to ergot agonists *in vivo*, also unrelated to an adenylyl cyclase; and (3) postsynaptic receptors closely associated with an adenylyl cyclase. Furthermore, in the substantia nigra there are: (4) presynaptic dopamine receptors (which are associated with adenylyl cyclase); and (5) autoreceptors (unassociated with adenylyl cyclase). From current knowledge, it is not possible to construct a theoretical framework ascribing a function, in terms of motor control, to any of these various dopaminergic mechanisms.

The concept of multiple categories of dopaminergic receptors has important implications for therapeutic medicine. Stimulation of D-1 receptors increases renal blood flow<sup>50</sup>. Dopaminergic agonists are currently used to treat circulatory shock; the pressor agents previously used had the disadvantage of producing renal vasoconstriction and thus decreasing the already compromised vascular perfusion of the kidneys.

Drugs which modify activity at the D-2 receptor have been exploited to treat endocrinological, neurological and psychiatric disorders. Bromocriptine, an agonist for the D-2 receptor, is the most satisfactory drug for stopping lactation in normal postpartum women or for pathological lactation in both women and men<sup>25,67</sup>. Up to 30% of women with secondary amenorrhoea and infertility have hyperprolactinaemia; in the majority of such patients, serum prolactin is reduced by bromocriptine, with consequent restoration of menstruation and fertility<sup>68</sup>. Bromocriptine has also been reported to decrease premenstrual tension in normal women<sup>69</sup> and to improve potency in men with hyperprolactinaemia<sup>68</sup>. In acromegalic subjects dopaminergic agents decrease the pathologically elevated levels of growth hormone; bromocriptine constitutes the first medical form of treatment for acromegaly, reversing the clinical consequences of abnormally high growth hormone concentrations<sup>70</sup>. The neurological features of parkinsonism seem to result from inadequate transmission at certain D-2 receptors. Bromocriptine has a therapeutic effect in the disease<sup>71</sup>, and the selective D-2 antagonist, molindone, induces the syndrome<sup>72</sup>. Dopaminergic blocking drugs are the cornerstone of treatment of schizophrenia; most of the agents involved are antagonists of both D-1 and D-2 receptors, but molindone, is an effective agent for treating schizophrenia<sup>72</sup>. In support of D-2 receptor involvement in schizophrenia, it is notable that bromocriptine can induce a florid paranoid psychosis<sup>71</sup>.

These examples suffice to indicate that if different categories of receptors mediate the various clinical results of manipulating dopaminergic activity, better treatment should be attainable by developing more specific drugs. At present there are no selective drugs for stimulating or inhibiting D-1 receptors in man, but recent animal studies suggest that such agents will be developed<sup>73,74</sup>. The desire to identify selective dopaminergic agonists and antagonists derives from the therapeutic successes which have been gained in the pursuit of such compounds for adrenoceptors, histamine receptors, and acetylcholine receptors.

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# articles

## Towards a theory for the uranian rings

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*Interparticle collisions, radiation drag and differential precession all tend to disrupt the rings of Uranus. The first two effects lead to radial spreading which would disrupt a free ring in  $\leq 10^8$  yr. We propose that the rings are confined in radius by gravitational torques from a series of small satellites that orbit within the ring system. Differential precession tends to destroy the apse alignment of the elliptical  $\epsilon$  ring. We suggest that apse alignment is maintained by the self-gravity of the ring. The resulting mass of the  $\epsilon$  ring is  $\sim 5 \times 10^{18}$  g. Its radial confinement requires (for example) a pair of satellites of mass  $\sim 10^{19}$  g, in circular orbits roughly 500 km away on either side of the ring.*

OCCULTATION observations have revealed that Uranus is surrounded by at least nine narrow rings<sup>1-4</sup>. The correct understanding of the origin and evolution of the rings has proved elusive. An early attempt to associate the five original rings with a series of orbital resonances involving known satellites was unsuccessful<sup>5-7</sup>. The subsequent discovery of four additional rings has made this approach less attractive.

The survival of narrow rings poses difficult dynamical problems.

### Dynamical problems

Unconstrained rings tend to spread due to particle collisions. The characteristic diffusion time  $t_D$  is comparable to the time a particle requires to random walk across the ring<sup>8,9</sup>,

$$t_D \sim t_c (\Delta r / \delta r)^2 \sim (\Delta r)^2 / \nu \quad (1)$$

where  $t_c$  is the collision time,  $\nu$  is the kinematic viscosity,  $\Delta r$  is the radial width of the ring and  $\delta r$  is the typical semi-major axis difference of colliding particles. Because the optical depth is at least of order unity,  $\Omega t_c \ll 1$ , where  $\Omega$  is the orbital angular velocity. The minimum value for  $\delta r$  is the mean particle radius  $d$ , so that  $\nu \geq d^2 \Omega$ . With parameters appropriate for Uranus' rings  $t_D \leq 2 \times 10^8$  (cm/d)<sup>2</sup> ( $\Delta r / 10$  km)<sup>2</sup> yr.

The Poynting–Robertson effect limits the lifetime of a ring composed of small particles. The torque on an optically thick ring of radius  $r$  due to radiation drag is

$$T_{PR} \sim - \frac{L_\odot \Omega r^3 \Delta r}{c^2 a_\odot^2} \quad (2)$$

where  $L_\odot$  is the solar luminosity and  $a_\odot$  is Uranus' semi-major axis. The orbital decay time for a ring of surface density  $\sigma$  is

$$t_{PR} \sim 4 \times 10^9 (\sigma / \text{g cm}^{-2}) \text{ yr} \quad (3)$$

For the two resolved rings ( $\beta$  and  $\epsilon$ ) the optical depth  $\tau \sim 1$  (refs 3, 4). Because  $\sigma \sim \rho d \tau$ , where the particles are composed of material of density  $\rho$ , the decay time is

$$t_{PR} \sim 4 \times 10^9 (\rho / \text{g cm}^{-3}) (d / \text{cm}) \text{ yr} \quad (4)$$

A ring with a range of particle sizes would spread in the much shorter time

$$t'_{PR} \sim t_{PR} (\Delta r / r) \sim 10^6 (\rho / \text{g cm}^{-3}) (d / \text{cm}) (\Delta r / 10 \text{ km}) \text{ yr} \quad (5)$$

where  $r \sim 5 \times 10^4$  km.

Combining the relationships for  $t_D$  and  $t'_{PR}$ , we find that the maximum survival time for a free ring is of order  $6 \times 10^6 (\rho / \text{g cm}^{-3})^{2/3} (\Delta r / 10 \text{ km})^{4/3}$  yr, and is obtained for a ring composed of particles with masses  $\sim (\Delta r / 10 \text{ km})$  kg. Therefore, if the rings are not very young, they must be confined. Three lines of evidence point to confinement. First, if the rings are young and unconfined, there should also be older, more diffuse rings visible in the occultation data, and these are not detected. Second, there is very little inter-ring material. A direct map of the rings at  $\lambda = 2.2 \mu\text{m}$  indicates that the total light reflected by inter-ring material is less than that reflected by the rings, implying a mean  $\tau \leq 10^{-3}$  between the rings<sup>10</sup>. Third, the  $\epsilon$  ring has very sharp edges, which implies that it is not spreading.

### Associated satellites

It is plausible that the rings are associated with small, as yet undiscovered, satellites. A satellite can resist tidal disruption at the location of the rings if it is either fairly dense,  $\rho_s \geq 2 \text{ g cm}^{-3}$ , or small,  $R_s \leq 100 \text{ km}$  ( $p_m / 10^7 \text{ dyn cm}^{-2}$ )<sup>1/2</sup> ( $\rho_s / \text{g cm}^{-3}$ )<sup>-1/2</sup>, where  $p_m$  is tensile strength ( $p_m \sim 10^7 \text{ dyn cm}^{-2}$  for ice and  $\sim 10^9 \text{ dyn cm}^{-2}$  for silicates or metals)<sup>11</sup>.

For the moment assume that both the satellite orbits and the rings are circular. Consider a satellite separated from the ring by a distance  $x$ . The satellite exerts a gravitational torque  $T_s$  which tends to repel the ring:

$$T_s \sim \pm f_1 \frac{G^2 m_s^2 r \sigma \Delta r}{\Omega^2 x^4} \quad (6)$$

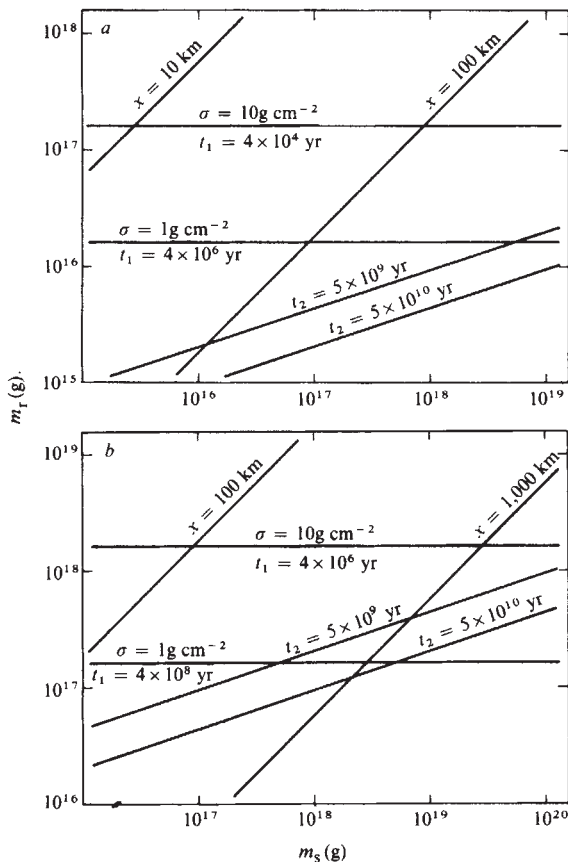
where  $m_s$  is the satellite mass,  $\Delta r$  is the ring width,  $\sigma$  is assumed to be uniform across the ring, and  $f_1$  is of order unity. The + and – signs apply if the satellite is inside or outside the ring respectively. Equation (6) may be obtained by a crude impulse approximation<sup>12</sup> or by summing the torques from all resonances of the form  $\Omega = n \Omega_s / (n \pm 1)$  which lie within the ring (ref. 13 and unpublished data).

It is valid for  $|\Delta r| \ll |x| \ll r$ . Diffuse material between two satellites will be driven into a ring on which the external torque is zero. The external torque deposits angular momentum in the inner part of the ring and removes it from the outer part. Angular momentum is transported outwards by shear stresses due to differential rotation and particle collisions. The ring will narrow until these two processes balance. In equilibrium

$$f_1 \frac{G^2 m_s^2 r \sigma \Delta r^2}{\Omega^2 x^5} \sim 2 \pi r^3 \sigma \nu \left| \frac{d\Omega}{dr} \right| \quad (7)$$

In equation (7) we assume that both satellites have the same mass. The left hand side is the magnitude of the net external torque on either half of the ring and the right hand side is the





**Fig. 1** Lines of constant satellite distance  $x$ , surface density  $\sigma$ , formation time  $t_1$  and disruption time  $t_2$  for rings of width: a, 5 km; and b, 50 km. If the satellites are trapped by resonances the value of  $t_2$  is irrelevant. The lines are plotted for  $f_1 = f_2 = 1$  and particle density  $\rho = 1 \text{ g cm}^{-3}$ . For fixed  $\Delta r$ ,  $m_r$  and  $m_s$ ,  $x \propto \rho^{2/5} (f_1/f_2)^{1/5}$ ,  $t_1 \propto \rho^2 f_2^{-1}$ ,  $t_2 \propto \rho^2 f_2^{-1}$ .

magnitude of the torque associated with the shear stress. As discussed earlier  $\nu \gg d^2 \Omega$ .

We now set  $\nu \sim f_2 \Omega (\sigma/\rho)^2$ . For a monolayer of particles with optical depth of order unity,  $f_2 \sim 1$ . We neglect the Poynting–Robertson torque, which is as important as the torque due to shear stresses for  $d \leq 10 \text{ cm}$ . We argue below that the average particle size in the  $\epsilon$  ring exceeds this limit.

Before viscous stresses become important, the ring narrows at rate  $d(\Delta r)/dt = \Delta r/t_1$ , where

$$t_1 \sim \frac{\Omega^3 r x^5}{f_1 G^2 m_s^2} \quad (8)$$

Using equation (7) it is easy to see that  $t_1$  is just the diffusion time  $t_D$  (see equation (1)) for the ring in its equilibrium state. This time is less than the age of the Solar System for a particle size  $d \geq 1 \text{ cm}$ .

If the satellites are unconstrained they recede from the ring. The characteristic time in which a satellite doubles its distance from the ring is

$$t_2 \sim (4m_s/m_r)t_1 \quad (9)$$

where  $m_r$  is the ring mass,

$$m_r = 2\pi\sigma r \Delta r \quad (10)$$

Sample solutions of equations (7)–(10) are shown in Fig. 1a and b. Not all regions of these diagrams correspond to allowed solutions. Our picture is only valid if several resonances from each satellite lie within the ring. This requires  $x \leq (r\Delta r)^{1/2} \sim 700 \text{ km}$   $(\Delta r/10 \text{ km})^{1/2}$ . A similar limit is suggested by the requirement that there is at least one satellite between every two rings, as there are at least nine rings in an interval of 10<sup>4</sup> km. If

the satellites are not constrained, we must have  $t_2 \leq 5 \times 10^9 \text{ yr}$ . However, the satellites may be trapped in resonances with the known satellites of Uranus. For the corotation resonances discussed previously<sup>5–7</sup> the satellites can be held in resonance if the dimensionless strength of the resonance satisfies

$$\mathcal{R} \geq f_1 \frac{G^2 m_s m_r}{r^2 \Omega^4 x^4} \quad (11)$$

Here  $\mathcal{R}$  is the amplitude of the resonance term in the disturbing potential in units of  $GM_\oplus/a$ . In general there are a number of resonances between the rings for which inequality (11) is satisfied<sup>7</sup>.

## Precessing ellipses

Another remarkable feature is that some of the rings are precessing ellipses. In particular, the inner and outer edges of the  $\epsilon$  ring can be fitted by aligned keplerian ellipses with semi-major axes  $a \pm \Delta a/2$  and eccentricities  $e \pm \Delta e/2$ , where  $a = 51,284 \text{ km}$ ,  $e = 7.8 \times 10^{-3}$ ,  $\Delta a = 60 \text{ km}$  and  $\Delta e = 7.2 \times 10^{-4}$ . The  $\alpha$ ,  $\beta$  and 4 rings also seem to be elliptical. The precession is presumably due to the quadrupole moment of Uranus. The observed rates for the  $\alpha$  and  $\epsilon$  rings together with the formula

$$(d\tilde{\omega}/dt)_0 = \frac{3}{2} J_2 n \left( \frac{R_\oplus}{a} \right)^2 \quad (12)$$

yield  $J_2 = 3.43 \times 10^{-3}$  for  $R_\oplus = 26,200 \text{ km}$  (ref. 4). Naive application of equation (12) predicts that the inner edge of the ring would precess by one revolution relative to the outer edge in 200 yr. Thus the observed alignment of the inner and outer edges must be explained.

The simplest explanation is that the apsides of the ring particles are locked together by the self-gravity of the ring. Consider two particles of mass  $m$  moving on aligned elliptical coplanar orbits, with orbital elements  $(a \pm \delta a/2, e \pm \delta e/2)$ , where  $0 < \delta a/a \ll 1$ ,  $|\delta e|/e \ll 1$ ,  $e \ll 1$ . It can be shown that as a result of their mutual gravitational interaction there is a contribution to the precession rate

$$\left( \frac{d\tilde{\omega}}{dt} \right)_G = \pm \frac{na}{\pi M_\oplus} \left[ (\delta a^2 - b^2)^{-1/2} \left( \frac{\delta a}{eb} - \frac{1}{2} \right) - \frac{1}{eb} \right] \quad (13)$$

Here  $b = a\delta e + e\delta a$  and we have assumed that the orbits do not cross, so that the quantity inside the square root is positive.

We can determine the mass  $m_r$  of the  $\epsilon$  ring by dividing it into a large number of subrings, applying equations (12) and (13) to calculate the precession rate of each subring, and requiring that all these rates are the same. A crude estimate of  $m_r$  is obtained by making two severe approximations: (1) only two subrings are used; (2), it is assumed that  $|b/\delta a| \ll 1$ . Then we find

$$\frac{m_r}{M_\oplus} = \frac{21\pi}{2} \frac{e}{\delta e} \left( \frac{\delta a}{a} \right)^3 J_2 \left( \frac{R_\oplus}{a} \right)^2 \quad (14)$$

Identifying  $\delta a$  and  $\delta e$  with half the total variation in semi-major axis and eccentricity across the  $\epsilon$  ring yields  $m_r = 1.1 \times 10^{19} \text{ g}$ . A more careful calculation using many subrings and the exact form of equation (13) yields  $m_r = 4.8 \times 10^{18} \text{ g}$ , corresponding to a mean surface density  $\sigma = 25 \text{ g cm}^{-2}$ . We have investigated the precession of rings with a wide variety of surface density profiles and find that  $m_r$  is insensitive to the details of the profile. Moreover, the eccentricity is always larger at the outer boundary than at the inner boundary, in accord with observations of the  $\epsilon$  ring.

We have considered three other potential mechanisms for maintaining apse alignment in a particle ring. First, the satellites which confine the ring may induce differential precession which cancels that due to the planet's quadrupole moment. However, application of equation (13) shows that the satellite's effect is negligible. Second, interparticle collisions give rise to an effective hydrodynamic pressure which modifies the particle orbits. It is easy to show that smooth pressure gradients are inadequate to overcome the differential precession. Third, a

discontinuity analogous to a hydrodynamic shock may form in the ring. A preliminary investigation suggests that, although the induced precession could be significant, it would not maintain rigid precession.

## Unanswered questions

Even if the model presented here is correct, there remain many unanswered questions. We have not discussed the origin of the ring material. The approximate equality of the masses of the rings and their confining satellites suggest that the rings may result from the collisional breakup of the satellites. A more speculative possibility is that the rings may reform into satellites.

Our confinement theory is strictly applicable only for circular rings and satellite orbits. Furthermore, we have not investigated the growth and decay of the eccentricities of the rings. It seems plausible that the satellite torques which confine a ring also tend to increase its eccentricity. On the other hand, dissipation due to shear stresses tends to decrease the eccentricity.

If we introduce the surface density deduced for the  $\epsilon$  ring into the equations for the confinement of circular rings (see Fig. 1b), we find that  $t_2$  is much less than the age of the Solar System. There are several possible explanations. The uncertainty in  $\rho$ ,  $f_2$  and  $x$  means that  $t_2 > 5 \times 10^9$  yr cannot be ruled out. Also, the confining satellites may be trapped in resonances. A confinement theory for elliptical rings might yield much larger

values of  $t_2$ . Finally, the time required for rings to form into satellites may be much less than the age of the Solar System.

The processes which determine the particle size distribution and optical depth in planetary rings are not understood. If there is a substantial population of small particles, the analysis must be modified to include the Poynting–Robertson effect.

The sharp edges of the  $\epsilon$  ring pose a challenge to any theory. They suggest that a portion of the constraining torques must be applied very near the ring boundaries.

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# UV spectrum of supernova remnant reveals carbon depletion in the interstellar medium

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*The first observation of the far UV, emission line spectrum of a supernova remnant (SNR), namely a filament in the Cygnus Loop, suggests that carbon, initially depleted in the gaseous phase by a factor of 10, is progressively returned to this phase, presumably by destruction of graphite grains in the high temperature, post-shock region.*

MODELS for the cooling and recombination of a collisionally ionised gas<sup>1,2</sup> such as is found in SNRs indicate that UV resonance lines of various elements dominate the emission spectrum in the UV–near IR region. In particular carbon, a key element in nucleosynthesis, has no prominent optical lines but emits strong UV lines in different stages of ionisation which can be used to determine accurate abundances.

Previous UV experiments have not looked at SNRs because of the extended nature and faintness of the filaments. We discuss here the first UV spectrum of a SNR obtained with the IUE satellite launched in January 1978 as a combined effort by NASA, ESA and the SRC. The IUE instrument and its performance are described elsewhere<sup>3</sup>.

One of the brightest filament of the Cygnus Loop, known as position 1 in a previous study in the optical region<sup>4</sup>, was selected for the observation. Filaments such as this are explained as being

due to radiative shocks of velocity 90–120 km s<sup>-1</sup> moving into a medium of density  $\sim 6$  cm<sup>-3</sup> (refs 2, 4, 5). This is consistent with the filamentary expansion velocity ( $\sim 120$  km s<sup>-1</sup>), but is much lower than that inferred from X-ray data. All observations<sup>6–8</sup> favour a plasma temperature of  $2\text{--}4 \times 10^6$  K which would imply a shock velocity of about 400 km s<sup>-1</sup>. The discrepancy has been explained as being due to denser cloudlets embedded in lower density material<sup>10</sup>.

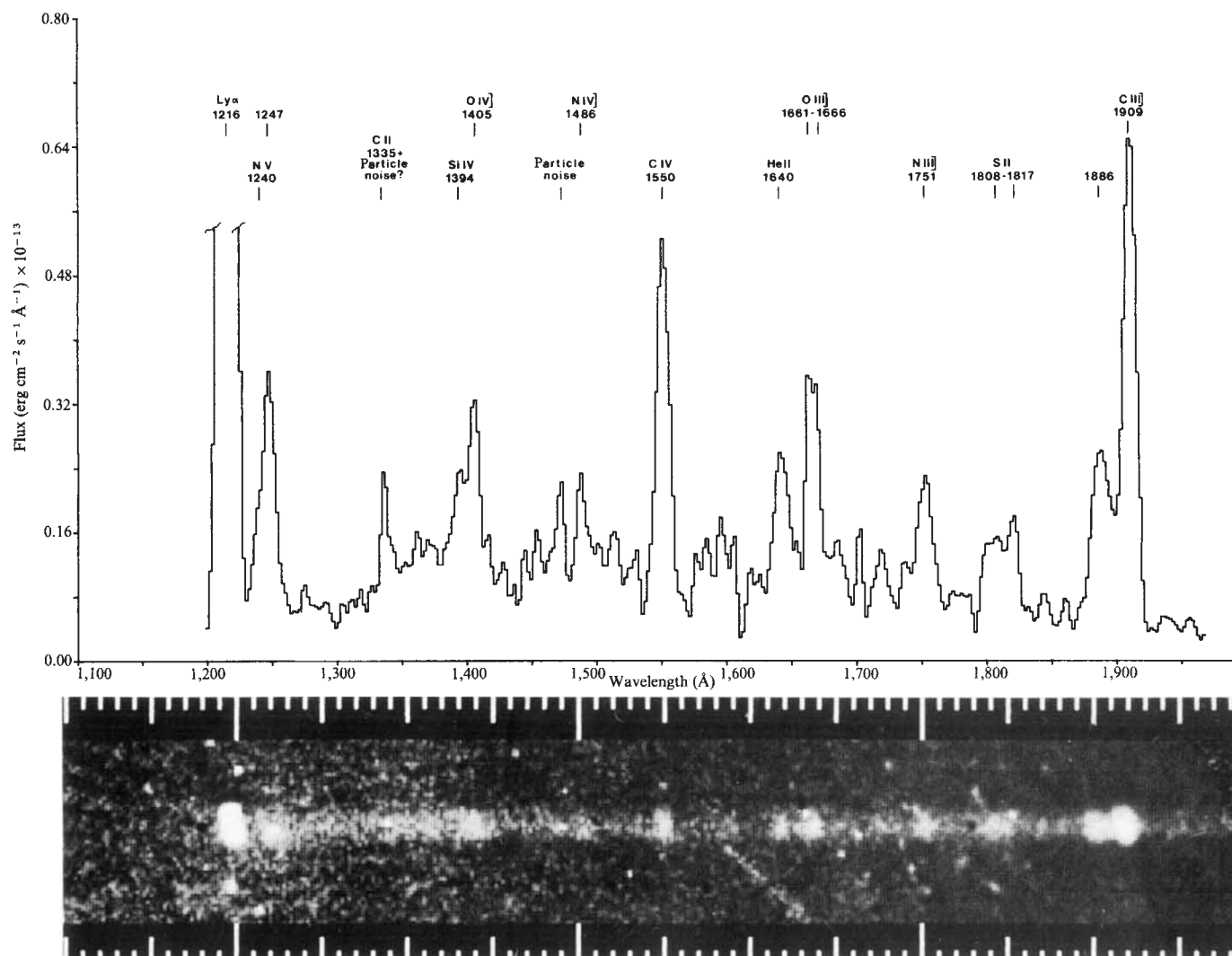
It is thought that the UV lines arise from shocks driving into these higher density cloudlets. Although no information can be obtained on the pre-shock density, the UV data can be used to derive a very accurate shock velocity, as described below.

## UV spectrum of a filament of the Cygnus Loop

A single, three-hour exposure was obtained in April 1978 in the 1,200–2,000 Å wavelength region using a blind offset procedure. Several emission lines are present including resonance transitions of C, N, O and Si plus the He II 1,640 Å emission. The strength of the geocoronal Ly $\alpha$  prevents any reliable measure of the Ly $\alpha$  emission from the Cygnus Loop. A continuum emission is detected along the full spectrum. The standard data reduction technique<sup>3</sup> was applied. At the time of writing an official absolute calibration for the IUE spectrographs was not available: to derive the relative intensities of the emission lines we have adopted a provisional calibration obtained by

\* Permanent address: Astrophysical Observatory, Asiago, Italy.





**Fig. 1** The UV spectrum of a filament in the Cygnus Loop in the 1,200–2,000 Å range and the resulting intensity plot with the identified emission lines. Several particle events appear as bright points in the three hours exposure.

R. Bohlin from spectra of the star  $\mu$  Col. As the errors are still uncertain, we will discuss the continuum distribution and the absolute line intensities elsewhere.

Figure 1 shows the reduced spectrum and the resulting intensity plot with the identifications of the emission lines. The spectrum has been smoothed and it is not corrected for interstellar absorption. The feature at 1,393 Å is probably a blend of Si IV 1,394 Å and S IV 1,385 Å, although the silicon should always be about four times stronger than sulphur. Two features at 1,247 Å and 1,886 Å are still unidentified: both seem too far from the plausible lines of Si II at 1,260 Å and Si III at 1,892 Å. Note that a small feature assigned to N V at 1,240 Å is really present even if it has disappeared from the smoothed tracing; its intensity has been measured from the original spectrum. The unidentified feature at 1,247 Å may be a diffuse particle event. Intensities relative to the C III 1,909 Å line are given in Table 1. The estimated error, due to uncertainty in the sensitivity curve and in some steps of the data extraction, is  $\sim 30\%$ .

### Physical conditions in the filament

The UV data can be used to derive a very accurate shock velocity. The reason for this is evident from consideration of shock models<sup>1,2,5,11</sup>. In the immediate post-shock gas, collisional ionisation increases the state of ionisation of the incoming gas, but, at the same time, cooling processes rapidly reduce the temperature so that the gas never has time to reach collisional ionisation equilibrium, although the maximum state of ion-

isation will follow the same sort of temperature dependence. Provided that hydrogen and helium are pre-ionised (which occurs<sup>11</sup> for shock velocities  $> 110$  km s<sup>-1</sup>), this maximum state of ionisation is just a function of the post-shock temperature.

As the gas cools rapidly, the temperature at which recombination occurs is effectively dependent only on atomic parameters, and is therefore independent of shock parameters. For species of very low ionisation potential such as C I and S I, the effects of photoionisation by the UV photons generated in the hot plasma become important and this conclusion is no longer valid.

With this simple model a semi-empirical function can be constructed for the ratio of two UV lines produced by ionic species,  $A^{+n}$  and  $A^{+(n+1)}$ , of the same atom. The ionisation balance between the ionic species will be of the form:

$$\frac{N(n+1)}{N(n)} = C_1 T^m \exp\left(-\frac{I_n}{KT}\right) \quad (1)$$

Where  $I_n$  is the ionisation potential of ion  $A^{+n}$  whose number density is  $N(n)$ . The power  $m$  depends on the recombination coefficient, but should be  $\sim 1.3$  for a wide range of atoms. The flux produced in UV lines is given by

$$F(n) = C_2 T^{-1/2} \bar{G}(T, Z) \exp(-E_n/KT) N(n) N_e \quad (2)$$

Where  $E_n$  is the excitation potential of the transition considered, and  $\bar{G}(T, Z)$  is a Gaunt factor of the order unity, a function of

both temperature and atomic charge  $Z$  (refs 12, 13). From equations (1) and (2) a fairly good approximation for the ratio of the two lines is:

$$\frac{F(n+1)}{F(n)} = C_3 T^m \exp\left(-\frac{I_n + E_{n+1} - E_n}{KT}\right) \quad (3)$$

In practice, the temperature of the zones producing the two lines will be somewhat different, but this will be ignored in the spirit of the approximation. Hence:

$$\ln \frac{F(n+1)}{F(n)} = \ln C_3 + m \ln T - \frac{I_n + E_{n+1} - E_n}{KT} \quad (4)$$

At low temperature, the last term on the right-hand side dominates, and  $T$  can be given by the post-shock temperature. In terms of shock velocity:

$$\left(\frac{150,000 \text{ K}}{T}\right) = \left(\frac{100 \text{ km s}^{-1}}{V}\right)^2 \quad (5)$$

from shock models. Hence, expressing velocities in units of  $100 \text{ km s}^{-1}$  and ionisation and excitation potentials in electron volts, at low shock velocity, the dimensionless function;

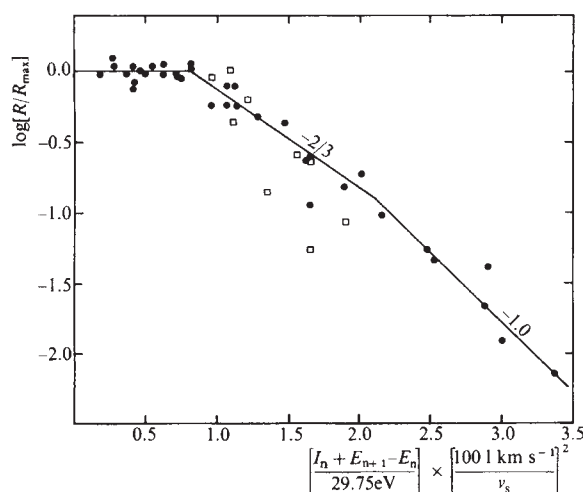
$$\log \frac{F_{n+1}}{F_n} = C_4 - \frac{I_n + E_{n+1} - E_n}{29.75 \text{ eV}} \left(\frac{100 \text{ km s}^{-1}}{V}\right)^2 \quad (6)$$

will correspond to a straight line of slope  $-1$ .

On the other hand, at high shock velocity the ratio of the two lines will tend to be constant because they are produced in two zones whose temperatures and thicknesses are in constant ratio. The graph should therefore become a horizontal straight line at high shock velocities.

At intermediate velocities, two effects working in opposite directions have to be taken into account. First, the mean temperature of the zone emitting the  $(n+1)$  species line does not increase as rapidly as in equation (5) when this zone becomes thick, but only half as rapidly. This would tend to produce a line of slope  $-0.5$ , but for the effect of the second term on the right of equation (4). For  $m = 1.3$  this tends to steepen the slope by  $-0.169$ , so at intermediate temperature, the curve should have a slope  $-0.669$  or about  $-2/3$ .

Figure 2 shows the universal curve so obtained for all temperature-sensitive ratios which reach saturation at the

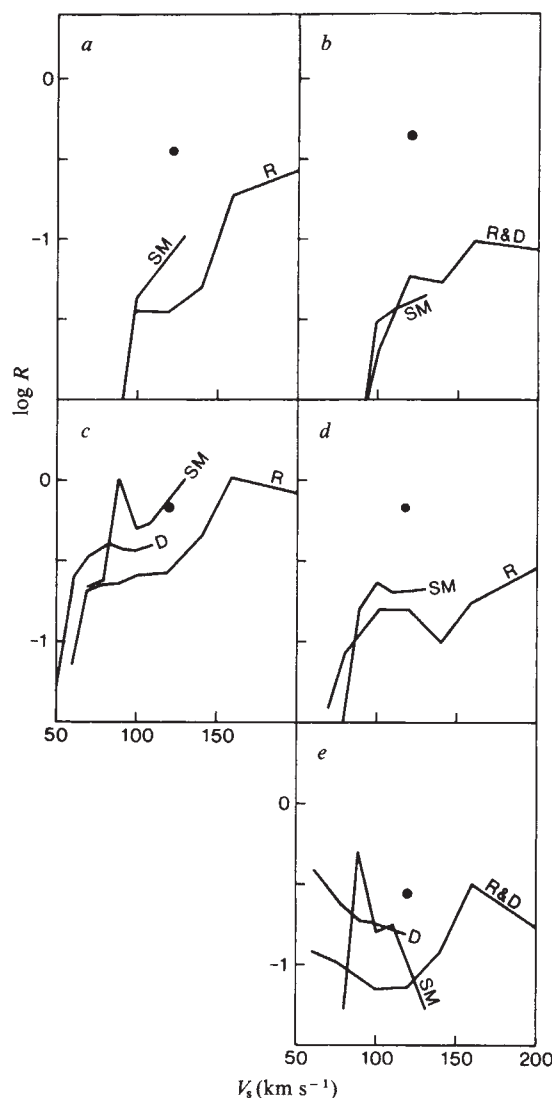


**Fig. 2** A plot of theoretical line intensity ratios versus a function of atomic constants and shock velocity. The ratios  $R$  are expressed in units of  $R_{\max}$ , the constant value which is reached at high velocities.  $I_n$  is the ionisation potential and  $E_n$  the excitation potential for the considered ion and transition respectively. ●, Represent ratios from models which assume full pre-ionisation over the whole velocity range<sup>2</sup>, whilst squares are from models with incomplete pre-ionisation.

**Table 1** Shock velocities for Cygnus Loop observations

| Ratio                                    | Shock velocity ( $\text{km s}^{-1}$ ) |
|------------------------------------------|---------------------------------------|
| O IV $\lambda$ 1406/O III $\lambda$ 1663 | 117–135                               |
| N V $\lambda$ 1240/N III $\lambda$ 1750  | 126–130                               |
| N IV $\lambda$ 1485/N III $\lambda$ 1750 | 102–118                               |
| C IV $\lambda$ 1549/C III $\lambda$ 1909 | 93–99                                 |
| C III $\lambda$ 1909/C II $\lambda$ 1335 | > 88                                  |

highest velocities<sup>2</sup>. Using this in conjunction with the shock models referred to above we derive the shock velocities for the Cygnus Loop observation given in Table 1. The carbon ratios give somewhat lower values than the rest; these are not used for reasons given below. We conclude that, for the region observed, the probable shock velocity is  $121 \pm 10 \text{ km s}^{-1}$ , in excellent agreement with the optical values.



**Fig. 3** The lines give the trend of various line intensity ratios as a function of shock velocity for different models (Raymond<sup>2</sup>(R), Shull and McKee<sup>11</sup>(SM), and Dopita<sup>1</sup>(D)) assuming standard abundances. The position of the Cygnus Loop observation (●) suggests an underabundance of all ions of carbon with respect to O, N and Si+S. a, O IV/C IV = 1,406/1,549; b, N IV/C IV = 1,485/1,549; c, O III/C III = 1,663/1,909; d, (Si IV + S IV)/C III = (1,394 + 1,385)/1,909; e, N III/C III = 1,750/1,909.



**Table 2** UV emission line intensities

| Line  | $\lambda$ (Å) | Observed | $X$ | Computed model |         | from  |           |
|-------|---------------|----------|-----|----------------|---------|-------|-----------|
|       |               |          |     | $F$            | $(F+G)$ | $F_d$ | $(F+G)_d$ |
| C II  | 1,909         | 100      | 100 | 100            | 100     | 100   | 100       |
| N III | 1,750         | 30       | 33* | 8*             | 12      | 20*   | 30        |
| O III | 1,663         | 68       | 128 | 28             | 83      | 70    | 207       |
| He II | 1,640         | 36       | —   | —              | 36      | —     | 90        |
| C IV  | 1,549         | 80       | 165 | 252            | 350     | 63    | 88        |
| N IV  | 1,485         | 34       | 13  | 14*            | 14      | 35*   | 35        |
| O IV  | 1,405         | 27       | 23  | 9              | 30      | 22    | 75        |
| Si IV | 1,393         | 36       | 2†  | 16†            | 22      | 40†   | 55        |
| S IV  | 1,335         | 48       | 34  | 42             | 51      | 66    | 81        |
| C II  | 1,335         | 48       | 34  | 42             | 51      | 66    | 81        |
| N V   | 1,240         | 31       | 2   | 2              | 14      | 5     | 35        |

$X$  is a carbon-depleted model with  $v_s = 100 \text{ km s}^{-1}$ ;  $F$  has no depletion and  $v_s = 110 \text{ km s}^{-1}$ ; both are from Raymond's work<sup>2</sup>.

$(F+G)$  is an average from two undepleted models of Shull and McKee<sup>11</sup>.  $F_d$  and  $(F+G)_d$  are synthesised models with carbon depleted in ratios.

1.0; 0.4; 0.2 in the logarithm for C IV, C III, and C II respectively.

\* Not given in the original computation. Estimated from calculated intensities of the same species with the help of Dopita<sup>1</sup> models.

† S IV not given in Raymond computation. Estimated from Shull and McKee<sup>11</sup> models.

## Determination of chemical abundances

As no hydrogen lines are seen, and the spectrum is dominated by the lines of carbon, we derive the abundance of ionic species with respect to the carbon species of closest ionisation potential. This ensures that the error made in mismeasurement of shock velocity is relatively unimportant.

The Cygnus Loop observation, when compared with the results of various shock models, reveals that all other elements (O, N and Si + S) are overabundant with respect to carbon. For the comparison with C III, this effect seems to be  $+0.4 \pm 0.2$  in the log, and by comparison with C IV the effect is larger, amounting to  $+1 \pm 0.15$  in the log. This effect can either be treated as an underabundance of carbon, or an overabundance of everything else. However, the former is the simplest hypothesis, and we adopt this here. In any event, observations of interstellar dark clouds<sup>14</sup> lead to underabundances of carbon in the gaseous phase of about a factor 10, very similar to the C IV value derived here which is ascribed to graphite grain formation.

In a radiating shock, such graphite grains would pass into the high-temperature zone, and if they are destroyed by the passage

through the radiating zone, this would show up as a progressive repletion of carbon in the gaseous phase towards lower temperature. This effect is precisely what is observed here, C IV being 0.6 (in the log) less abundant than C III. At risk of overinterpreting the data, the C II/C III  $\lambda 1335/\lambda 1909$  ratio the data suggests a depletion of  $0.2 \pm 0.2$  for C II.

Introducing depletions of 1.0:0.4:0.2 (C IV:C III:C II) makes all the data consistent. In particular, the C IV/C III  $\lambda 1549/\lambda 1909$  ratio gives a low shock velocity without differential depletion. When this is included, however, the shock velocity derived from this ratio is in the range  $110\text{--}123 \text{ km s}^{-1}$ , in full agreement with the other values.

This internal consistency is reflected in full shock model calculations summarised in Table 2. The Raymond model<sup>2</sup>  $X$ , used to reproduce the optical spectrum, had a velocity  $V_s = 100 \text{ km s}^{-1}$  with a depletion of both carbon and silicon. Although this model is a reasonable fit for many lines, it fails for the high excitation ones (such as N IV, O IV) indicating a too low velocity. Higher velocity models without depletion,  $F$  and  $(F+G)$ , fail by predicting too strong C IV and other lines somewhat too weak. Only for the differentially depleted models is there a good fit, with all lines fitting the observations to within a factor of about two, which, bearing in mind the uncertainties in the atomic physics and modelling procedures, is about as good as could possibly be achieved at present.

## Conclusion

There is very strong evidence that carbon, initially depleted in the gaseous phase by a factor of  $\sim 10$ , is progressively returned to this phase through the cooling zone, presumably by destruction of graphite grains. The mechanism of this process will be discussed in full in a later paper.

This work was based on observations by the IUE collected at the Villafranca Satellite Tracking Station of the ESA.

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# Tholins: organic chemistry of interstellar grains and gas

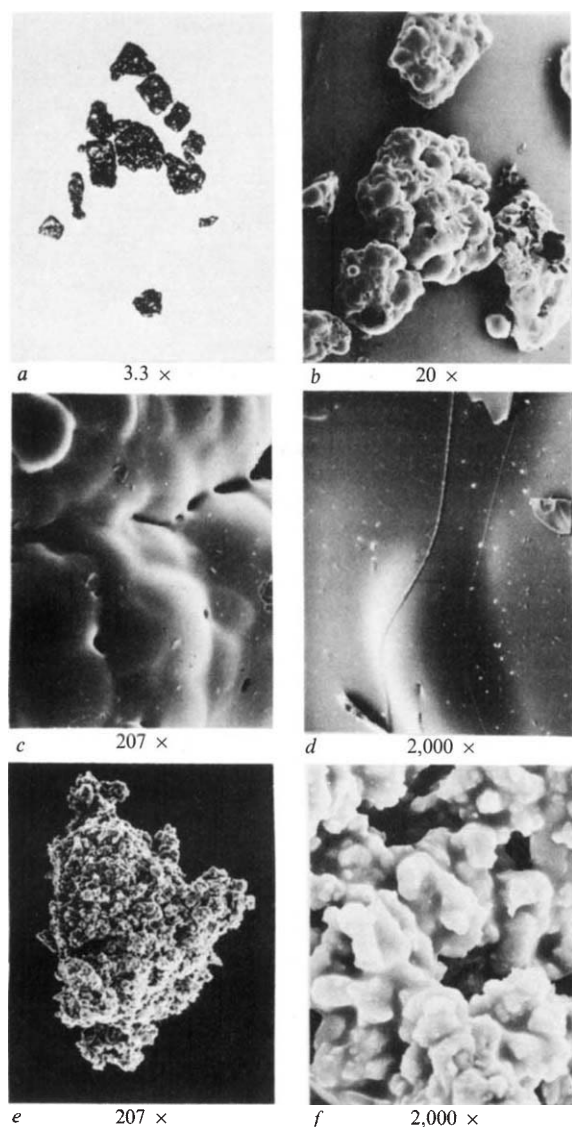
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*A complex organic solid produced from cosmically abundant molecules helps to explain many properties of the interstellar grains and gas.*

FOR the past decade we have been producing<sup>1–4</sup> in our laboratory a variety of complex organic solids from mixtures of the cosmically abundant gases  $\text{CH}_4$ ,  $\text{C}_2\text{H}_6$ ,  $\text{NH}_3$ ,  $\text{H}_2\text{O}$ ,  $\text{HCHO}$ , and  $\text{H}_2\text{S}$ . The product, synthesised by ultraviolet (UV) light or spark discharge, is a brown, sometimes sticky residue, which has been

called, because of its resistance to conventional analytical chemistry, 'intractable polymer'. However, we have recently succeeded, through sequential and non-sequential pyrolyses followed by gas chromatography-mass spectrometry (GC-MS), in determining something of the composition of this material<sup>3,4</sup>. It is clearly not a polymer—a repetition of the same monomeric unit—and some other term is needed. We have discussed this material as a constituent of the Earth's primitive oceans and therefore as relevant to the origin of life<sup>1</sup>; as a component of red aerosols in the atmospheres of the outer planets and Titan<sup>2,5</sup>; as



**Fig. 1** Light microscopy (a) and scanning electron microscopy (b–d) of spark tholins heated to 920 °C. The smallest particles in (d) are the size of interstellar grains. Also shown (e–f) is the quite different gross structure of UV tholins without elemental S, probably rich in disulphide bridges.

present in comets, carbonaceous chondrites, and pre-planetary solar nebulae<sup>2,5</sup>; and as a major constituent of the interstellar medium, which is the concept discussed here. We propose, as a model-free descriptive term, 'tholins' (gk θόλος, muddy; but also θολός, vault or dome), although we were tempted by the phrase 'star-tar'. The properties of tholins will depend on the energy source used and the initial abundances of precursors, but a general physical and chemical similarity among the various tholins is evident.

UV tholins are produced by near-UV photodissociation of  $\text{H}_2\text{C}$  or  $\text{HCHO}$ , and chain reactions involving the resulting superthermal H atom. Spark tholins, first made by S. L. Miller<sup>6</sup>, are produced by electrical discharges. Both involve the high-temperature dissociation of precursor gases and subsequent low-temperature recombination of radicals. A comparison of the two tholins which we have studied most is presented in Table 1. The 50 or so identified pyrolytic fragments in each tholin extend to molecules as complex as  $\text{C}_3$ -alkylbenzenes,  $\text{C}_4$ -alkylthiophenes,  $\text{C}_6$ -alkyldisulphides, and  $\text{C}_5$ -alkylthiocyanates in UV tholins; and  $\text{C}_4$ -alkylbenzenes,  $\text{C}_2$ -alkylindanes, methylethylpyrazine, glutaronitrile, and  $\text{CH}_2=\text{CH}-\text{CH}=\text{CHCH}_2\text{CH}_2\text{CH}_3$  (heptadiene) in spark tholins. (A full list of identified fragments can be found in refs 3 and

4.) There is evidence of amino acid precursors—perhaps polypeptides—in both tholins<sup>4</sup>, and a variety of biologically interesting amino acids, including cystine, have been isolated on tholin hydrolysis<sup>1</sup>. The overall tholin structure may be a complex matrix, dotted with aromatic hydrocarbons, pyrroles, pyridines and other rings, and crosslinked with long-chain alkanes, alkenes and polypeptides, somewhat similar to the structure of kerogen or coal. Scanning electron micrographs of tholins are displayed in Fig. 1. The highest molecular weight fragments which have been identified are about 150 daltons, but fragments at 500 to 700 daltons have been observed and still heavier fragments obviously exist. The TGA thermal stability, especially for the spark tholin (Table 1), is impressive; the tholins have experienced a kind of stability natural selection during their synthesis in a UV or high energy proton environment. In their production, the UV tholins received a UV dose exceeding the interstellar near-UV energy density integrated over the lifetime of the Galaxy. The greater complexity of spark over UV tholins at highest magnifications (Fig. 1d, 1f) may be consistent with the higher stability of the former. We argue here that the tholins are a major constituent of interstellar grains and that their spallation products—similar to the pyrolytic GC-MS fragments—are the primary source of the organic molecules found by microwave line spectroscopy in the interstellar gas.

**Table 1** Comparison of typical UV and spark discharge tholins

|                                           | UV <sup>3</sup>                                                                                                                | Spark <sup>4</sup>                                                                                                             |
|-------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------|
| Initial reactants                         | $\text{CH}_4$ , $\text{C}_2\text{H}_6$ , $\text{NH}_3$ , $\text{H}_2\text{O}$ , $\text{H}_2\text{S}$ (roughly equimolar)       | $\text{CH}_4$ , $\text{NH}_3$ , $\text{H}_2\text{O}$ (~2.5%)                                                                   |
| Energy source                             | 2,537 Å Hg lamp                                                                                                                | High-frequency tesla coils                                                                                                     |
| TGA, 50% thermal dissociation temperature | ≈ 300 °C                                                                                                                       | ≈ 900 °C                                                                                                                       |
| Atomic composition                        | Apart from S:<br>H, 3.8%; C, 37%; N, 11%; O, 48%                                                                               | H, 5.0%; C, 47%<br>N, 36%; O, 12%                                                                                              |
| Principal GC-MS Pyrolytic products        | Alkanes, alkenes, aromatic hydrocarbons, alkylthiophenes, alkylmercaptans, alkyldisulphides, isothiocyanates and some nitriles | Alkanes, alkenes, abundant nitriles, aromatic hydrocarbons, alkylbenzenes, indenes, indanes, pyrroles, pyridine, and pyrazines |

The conventional composition assigned to interstellar grains—on the basis of their UV and IR spectra, optical frequency extinction, polarisation data and cosmic abundances—is a mixture of silicates, water ice, graphite, carborundum and iron<sup>7-9</sup>. The silicate and ice identifications are probably secure, on cosmic abundance grounds and also, for example, because interstellar features attributed to silicates are also seen in the circumstellar envelopes of oxygen-rich red giant stars. However, it has long been recognised that complex organic molecules provide a possible explanation for several otherwise puzzling properties of the interstellar medium. Sagan<sup>8</sup> argued that the *in situ* formation of the more complex interstellar gas molecules was very difficult to understand, and suggested instead that they are degradation products of the interstellar grains, which he proposed included stable complex organics, ejected from solar nebulae by radiation pressure and stellar winds in the early stages of evolution of stars later than spectral-type F. The fact that the abundances of interstellar organics do not decline steeply with heavy atom number points strongly to an origin as dissociation products of more complex organic species rather than an *in situ* synthesis from interstellar



**Table 2** Proposed origin of interstellar molecules (All interstellar molecules composed of C, H, N, and O, known as of mid-1978, are listed; column numerals represent heavy atom number)

| Molecule                      |                                     | Relevant laboratory experiment |                                 |                              |                           | Other*                                             |
|-------------------------------|-------------------------------------|--------------------------------|---------------------------------|------------------------------|---------------------------|----------------------------------------------------|
|                               |                                     | Gas phase spark <sup>30</sup>  | Interstellar frost <sup>9</sup> | Spark pyrolysis <sup>4</sup> | UV pyrolysis <sup>3</sup> |                                                    |
| 1. Hydrogen                   | H <sub>2</sub>                      |                                | X                               | X                            | X                         | Precursor gas                                      |
| Methane                       | CH <sub>4</sub>                     | X                              |                                 | X                            | X                         | Precursor gas                                      |
| Methylidyne                   | CH, CH <sup>+</sup>                 | X                              |                                 | X                            | X                         | Any hydrocarbon                                    |
| Ammonia                       | NH <sub>3</sub>                     | X                              | X                               | X                            | X                         | Precursor gas                                      |
| Amino radical                 | NH <sub>2</sub> <sup>+</sup>        | X                              |                                 | X                            | X                         | NH <sub>3</sub> , any amine                        |
| Water                         | OH <sub>2</sub>                     | X                              |                                 | X                            | X                         | Precursor gas                                      |
| Hydroxyl                      | OH                                  | X                              |                                 | X                            | X                         | Water                                              |
| 2. Ethynyl                    | C≡CH                                |                                |                                 | X                            | X                         | Any acetyl or ethynyl group                        |
| Acetylene                     | HC≡CH                               | X                              | X                               |                              |                           |                                                    |
| Methylamine                   | CH <sub>3</sub> NH <sub>2</sub>     |                                |                                 |                              | X <sup>2</sup>            | Tholin amino cmpds. (see text)                     |
| Methyleneimine                | CH <sub>2</sub> =NH                 |                                |                                 | X                            | X                         | CH <sub>3</sub> NH <sub>2</sub> - H, HNC + H, etc. |
| Cyanogen                      | C≡N                                 |                                |                                 | X                            | X                         | Any nitrile                                        |
| Hydrogen cyanide & isocyanide | HCN, HNC                            | X                              | X                               | X                            | X                         | Many nitriles                                      |
| Carbonyl                      | C=O                                 | X                              |                                 | X                            |                           |                                                    |
| Formyl                        | HCO, HCO <sup>+</sup>               |                                |                                 | X                            | X                         | Any aldehyde                                       |
| Formaldehyde                  | HCHO                                | X                              | X                               |                              |                           |                                                    |
| Methanol                      | CH <sub>3</sub> OH                  |                                | X                               |                              |                           |                                                    |
| 3. Methylacetylene            | CH <sub>3</sub> -C≡CH               |                                |                                 |                              |                           | Propene - H, etc.                                  |
| Acetonitrile                  | CH <sub>3</sub> C≡N                 | X                              | X                               | X                            | X                         |                                                    |
| Cyanamide                     | NH <sub>2</sub> C≡N                 |                                |                                 |                              |                           | Tholin amino cmpds. (see text)                     |
| Acetaldehyde                  | CH <sub>3</sub> CHO                 | X                              | X                               | X                            |                           |                                                    |
| Formic acid                   | HCOOH                               |                                | X                               |                              |                           |                                                    |
| Formamide                     | NH <sub>2</sub> CHO                 |                                |                                 |                              |                           | Tholin amino cmpds. (see text)                     |
| Ketene                        | CH <sub>2</sub> =C=O                |                                |                                 |                              |                           | Acetaldehyde - H, etc.                             |
| Isocyanic acid                | HN=C=O                              |                                |                                 |                              |                           | Formamide - H (note analogy to isothiocyanates)    |
| Ethanol                       | CH <sub>3</sub> CH <sub>2</sub> OH  |                                | X                               |                              |                           |                                                    |
| Dimethylether                 | (CH <sub>3</sub> ) <sub>2</sub> O   |                                |                                 |                              |                           | Methanol + CH <sub>2</sub>                         |
| 4. Cyanoethynyl               | C≡C-C≡N                             |                                |                                 |                              |                           | Acrylonitrile - H, Propylnitrile - H, etc.         |
| Cyanoacetylene                | HC≡C-C≡N                            |                                |                                 |                              |                           | Acrylonitrile - H, etc.                            |
| Acrylonitrile                 | CH <sub>2</sub> =CHCN               |                                |                                 | X                            | X                         |                                                    |
| Methylformate                 | CH <sub>3</sub> COOH                |                                | X                               | X                            |                           |                                                    |
| Tropionitrile                 | CH <sub>3</sub> CH <sub>2</sub> C≡N |                                |                                 | X                            |                           |                                                    |
| 6. Cyanodiacetylene           | CH≡C-C≡C-C≡N                        |                                |                                 |                              |                           | Pentenitrile - H, etc.                             |
| 8. Cyanoctatetrayne           | CH≡C-C≡C-C≡C-C≡C-C≡C-C≡N            |                                |                                 |                              |                           | Benzonitrile ring breakage - H                     |

(Table courtesy of L. E. Snyder.)

\* All molecules cited in this column are identified either in tholin pyrolyzates or in the interstellar medium.

gas. If the presumed dissipation of the preplanetary solar nebula in our Solar System is typical of main sequence stars generally, a significant contribution<sup>10</sup> of organics to the interstellar medium is made, probably enough to coat silicate cores formed, for example, in circumstellar shells (see ref. 40). He suggested that the composition of the interstellar grains might be similar to that in contemporary carbonaceous chondrites and cometary nuclei. Because interstellar grains might be abundant in preplanetary solar nebulae, and contribute to carbonaceous chondrite chemistry, we note the possibility of multiple recycling of stable organic matter between circumstellar disks and interstellar clouds.

Alternatively, we have shown experimentally<sup>9</sup> that near-UV irradiation at 77 K of stacked layers of HCHO, H<sub>2</sub>O, NH<sub>3</sub> and C<sub>2</sub>H<sub>6</sub> on a silicate matrix produced, on vapourising the frosts, a variety of simple organic molecules similar to those found by microwave spectroscopy in the interstellar medium, and suggested that organic synthesis also occurs *in situ* on ice-coated grains of silicates or other materials. For both modes of synthesis, Sagan<sup>10</sup> proposed that labile molecules would be preferentially destroyed, and that the steady state molecular population of the grains must, through natural selection, be exceptionally stable complex organics. Because of the Frank-Rabinowitch principle on the stability of multiply cross-linked structures, unbranched aliphatics, say, are not expected to be dominant, while polycyclic aromatics, complexly branched

aliphatics and a matrix of mixed rings and aliphatics would be much more likely. Subsequently, Knacke<sup>12</sup> argued that complex organics, including one of our UV tholins, might help explain several unidentified or puzzling interstellar IR absorption or emission features, but was unable to secure an entirely acceptable fit.

Since this early work, very similar ideas seem to have been taken up by Hoyle and Wickramasinghe<sup>13-17</sup>, who have paid particular attention to the growing body of data on IR absorption and emission in the interstellar medium. They showed that organic polymers display absorption in the 3 and 8 to 12 μm regions which roughly match the interstellar absorption. In fact, a very wide range of mixtures of complex organic molecules will satisfy such unrestrictive absorptivities, because the 3 μm absorption corresponds to characteristic vibrational transitions of O-H, N-H and, particularly, C-H bonds, while the 8-12 μm region is characteristic of C-C, C-O and C-N functional groups. But they then went on to attempt a more detailed match of organics with interstellar absorption, and claim to have 'identified' the formaldehyde polymer polyoxymethylene and, later, polysaccharides as major constituents of interstellar grains; and have gone still further in postulating sporopollenin, a particular category of polysaccharides found in the cell walls of spores and pollen<sup>18</sup>, with typical empirical formulae ranging from C<sub>90</sub>H<sub>115</sub>O<sub>10</sub> to C<sub>90</sub>H<sub>158</sub>O<sub>44</sub>, as a reasonable candidate for the composition of interstellar grains.

However, no plausible mechanism for producing such a polysaccharide in the interstellar medium or in circumstellar envelopes has been offered. They are not abundant products in typical experiments on prebiological organic chemistry which involve the irradiation or heating of cosmically abundant substances. They and their pyrolyzates do not appear, to the limit of experimental detectivity, in our analysis of tholins, including experiments in which  $\text{H}_2\text{O}$  is in excess. Polysaccharides are composed only of the atoms H, C and O and therefore do not reflect the high cosmic abundance of N and S, which, as the composition of tholins clearly illustrates, should be major constituents of organic molecules produced from cosmically abundant gases. Moreover, the idea that any particular molecule such as sporopollenin, or even a particular class of organic molecules such as the polysaccharides, should be typical of interstellar grains seems very unlikely; laboratory experience suggests that a very wide range of products should instead be produced under all plausible production scenarios.

There is no such thing as a common IR spectrum characteristic of all galactic sources. Absorption features are well-known in many sources near 3, 10 and 20  $\mu\text{m}$ , but with significant variability from source to source (and in some cases even with time) in the band central wavelengths, depths and half widths. Additional absorption and emission features are known in some sources at 3.3, 6.0, 6.2, 6.8, 7.7, 8.6, 11.3  $\mu\text{m}$  and other wavelengths. The very variability of IR spectra from source to source implies that a variety of molecules are responsible. On cosmic abundance grounds and on the basis of detailed models it seems likely that the 3.1, 9.7, and 18.5  $\mu\text{m}$  features can be explained, perhaps in all cases, by water or ammonia ices and silicates. (Features attributed to ices are very rare in the general interstellar medium and are apparently found only in molecular clouds<sup>19,20</sup>). But even for these features many problems exist. In the source OH 26.5 + 0.6 the 3.1  $\mu\text{m}$  feature is unlikely to be ice because the grain temperatures are too high although it may be due to gas phase ( $\text{C}_2\text{H}_2 + \text{HCN}$ ) molecular absorption in a cool stellar atmosphere; also the grains show appreciable opacity shortwards of 7  $\mu\text{m}$  where silicates are generally transparent<sup>21</sup>. In compact H II regions and NGC7027 there is a strong emission feature at 3.3  $\mu\text{m}$  which cannot be due to ice, in turn invoked for H II regions to explain the adjacent 3.08  $\mu\text{m}$  feature<sup>22</sup>. In OH231.8 + 4.2  $\equiv$  OH0739 - 14 a deep feature at 3.08  $\mu\text{m}$  still has substantial wings at 2.3  $\mu\text{m}$ , inconsistent with ice; in addition, the relative extinctions at characteristic wavelengths for silicates and ice are inconsistent with known oscillator strengths and cosmic abundances. Likewise the band shapes and depths of features attributed to water ice and silicates in the galactic centre<sup>24</sup> and in the Becklin-Neugebauer Object<sup>13-17,25</sup> in the Orion Nebula are inconsistent with cosmic abundances. The spectra of compact H II regions at 20 to 33  $\mu\text{m}$  are not readily understood in terms of their 10  $\mu\text{m}$  absorptions if silicates are invoked for both<sup>26</sup>. Some of the foregoing difficulties may be resolvable with tholins as additional constituents in the interstellar medium.

Figures 2-4 display the IR spectra of UV and spark tholins, and a few of their extracts and high-temperature phases, compared with a variety of other materials—including a sporopollenin; a thin layer chromatography cellulose matrix, as an example of non-sporopollenin polysaccharides; carbonaceous chondrites<sup>27,28</sup>; and two galactic spectra, one of which is Hoyle's 'best fit' to many galactic IR sources<sup>29</sup>. Spark and UV tholins exhibit substantial family resemblances in the IR; some of the differences are due to S compounds present in the latter and not the former. Heating tholins to high temperatures seems to destroy short wavelength absorption bands and increase continuum absorption. Functional groups are well-known to shift band central wavelengths by several tenths of a  $\mu\text{m}$  depending on physical state and adjacent chemistry. Some tholin IR absorptions may be due to collective phenomena (long polymers, for example, having different spectra from monomers); tholin spectra will vary from source to source

depending on precursor abundances and energy sources at their origin, heating and irradiation during ejection from circumstellar clouds, and other effects. We believe that mixes of tholins, such as that in Fig. 3, match the galactic spectra at least as well as sporopollenin, and exhibit the additional virtue of having a plausible synthetic mechanism. The fit of four spectral features between 3 and 40  $\mu\text{m}$  will occur by chance with a probability  $10^{-8}$ . We anticipate still better agreement with interstellar spectra for mixes of tholins, ices and silicates.

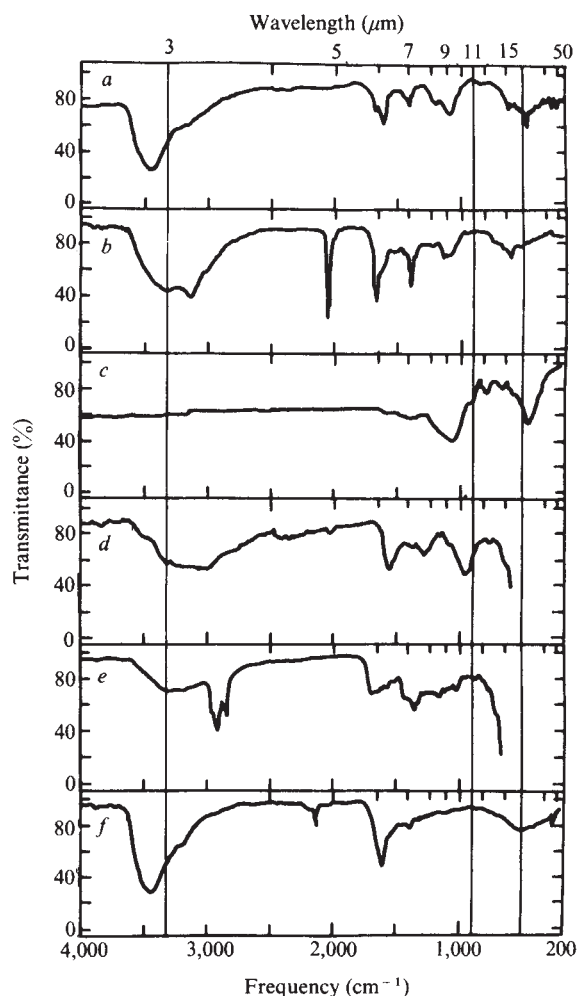
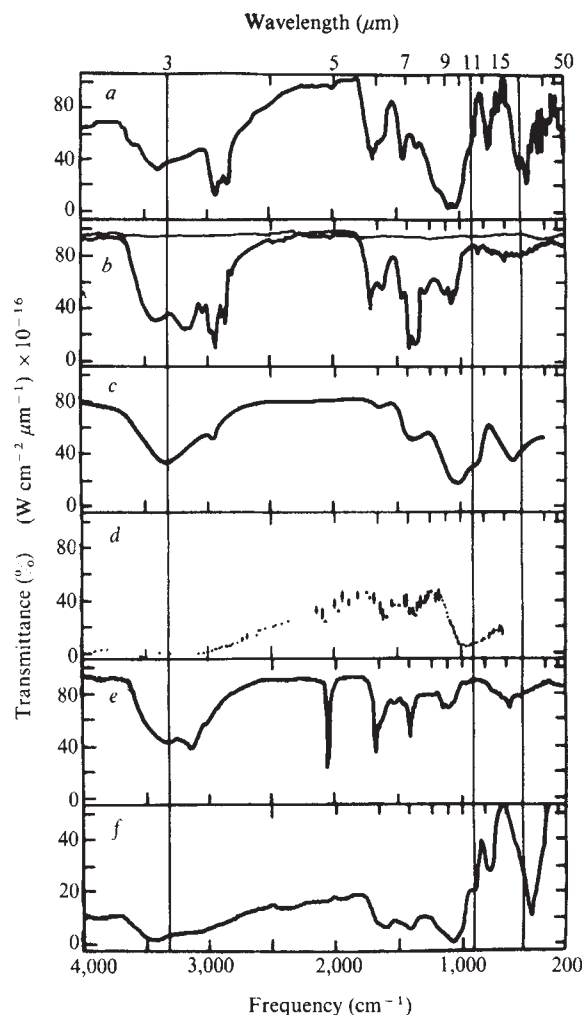


Fig. 2 Comparative IR spectra of a, UV tholin; b, UV tholin, elemental S removed; c, UV tholin, elemental S removed, heated to 450 °C; d, methanol extract of UV tholin; e, methanol extract of chromatographically separable yellow component of UV tholin; f, spark tholin. Vertical lines 3, 11 and 18  $\mu\text{m}$  are for reference.

In NGC7538E, a compact IR source associated with a rich molecular cloud complex, there are unidentified features<sup>30</sup> at 6.0 and 6.8  $\mu\text{m}$  which correlate with the presence of the 3.1  $\mu\text{m}$  feature, but not the 9.7  $\mu\text{m}$  feature. But in our early work on the IR spectra of UV tholins we noted<sup>2</sup> the presence of features at 3.42 and 6.87  $\mu\text{m}$  due to long-chain alkanes, and near 6.0  $\mu\text{m}$  due to alkenes. In fact the presence of these features was a major early clue that long-chain alkanes and alkenes are an important constituent of tholins, a conclusion since confirmed by GC-MS<sup>3,4</sup>. Willner *et al.*<sup>30</sup>, who discovered the 6.0 and 6.8  $\mu\text{m}$  features in NGC7538E, hold that a problem with the alkane and alkene identification is that the ratio of gas phase CO to grain  $\text{CH}_2$  or  $\text{CH}_3$  would have to be as high as 1/3; but such groups are the dominant functional groups in all analysed tholins. If the





**Fig. 3** Comparative IR spectra of *a*, a sporopollenin<sup>13-17</sup>; *b*, TLC cellulose; *c*, Hoyle's<sup>29</sup> galactic 'best fit'; *d* NGC 7538E<sup>30</sup>; *e*, UV tholin-S; *f*, linear superposition of (*a*), (*c*) and (*d*) of Fig. 2. The transmission in the 760 to 200  $\text{cm}^{-1}$  region in Fig. 2*d* is added at the 100% level.

mantles of interstellar grains are made significantly of tholins, the high methyl and methylene abundances become plausible. There is also a hint in the spectra of NGC7538E of absorption at 4.6  $\mu\text{m}$ , which is characteristic of  $\text{C}\equiv\text{C}$  and  $\text{C}\equiv\text{N}$ . Features near 6.2, 7.7, 8.6 and 11.3  $\mu\text{m}$  first found<sup>31</sup> in NGC7027—and since found<sup>30</sup> in many other regions of the Galaxy, as well as in M82—have analogous absorptions in several tholins (Figs 2 and 3). The presence of 8.7 and 11.3  $\mu\text{m}$  features in the young IR source GL437 implies that they are not produced by anomalously abundant elements which might be present in highly evolved objects<sup>32</sup>, but rather must be attributed to typical cosmic abundances, consistent with organic molecules.

It is a natural speculation that tholins are present in carbonaceous chondrites, and we<sup>2</sup> and Knacke<sup>12</sup> have compared relevant IR spectra. The agreement is good but not excellent for samples of Murray and Orgueil. We note here (see Fig. 4) a quite good agreement between a particular (hot-water soluble, cold-water insoluble) fraction of the Mokoia carbonaceous chondrite<sup>27,28</sup> and Hoyle's 'best fit' galactic spectrum<sup>29</sup>. However, the chemistry of Orgueil, determined<sup>33</sup> under identical conditions of pyrolytic GC-MS as the tholin analysis, demonstrates a relative dearth of S and N compounds (especially nitriles) in Orgueil. Depolymerisation of the Murchison carbonaceous chondrite reveals a range of organic products very similar to that of S-free tholins<sup>33</sup>. More work is clearly needed

on the comparative organic chemistry of carbonaceous chondrites, and, in particular, of the appropriate fraction of Mokoia.

The principal UV interstellar absorption feature is at 2,200 Å, conventionally attributed to graphite, which should be formed and ejected by C-rich red giant stars. However, the graphite identification requires that its structure be well-ordered, which is unlikely in the interstellar radiation environment. Large sheets of polycyclic aromatic hydrocarbons have similar absorption features, but are subject to the same objection. Sakata *et al.*<sup>34</sup> have found a 2,200 Å feature in a hexafluoroisopropanol extract of the Murchison carbonaceous chondrite and attribute its presence to organic molecules with conjugated double bonds of the sort  $\text{C}=\text{C}-\text{C}=\text{C}$  and  $\text{C}=\text{C}-\text{C}\equiv\text{N}$ . But such molecules are abundant in tholins, as in butadiene, heptadiene and butenenitrile. Polysaccharides, on the other hand, exhibit no such feature<sup>35</sup>.

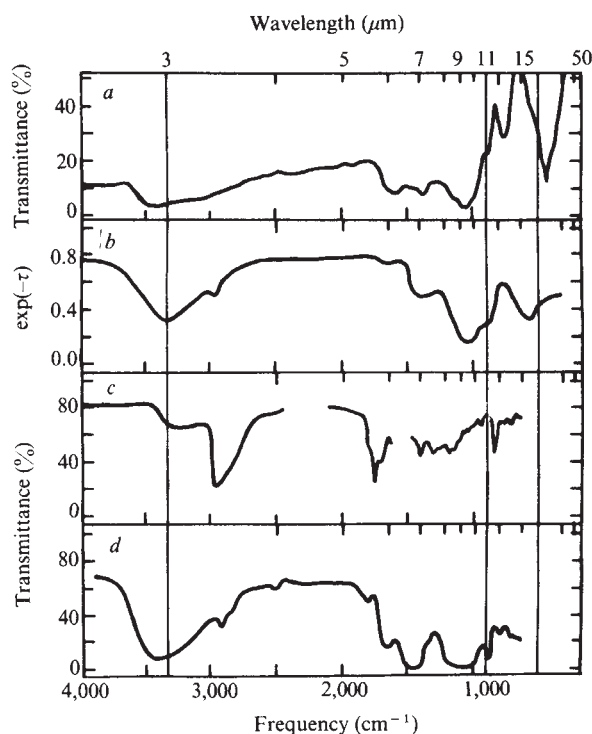
The optical region of the interstellar spectrum is noteworthy for the presence of a set of diffuse interstellar lines, the most prominent at 4,428 Å, which have resisted identification for 40 years. The hypothesis that 4,428 Å is the Soret band of porphyrins (tetrapyrroles) is interesting in the light of our identification of pyrrole in spark tholins, but there are serious difficulties with this idea<sup>10</sup>. Douglas<sup>36</sup> has recently argued that the diffuse lines arise from radiationless internal conversion of long-chain C molecules, where the carbon number lies between 5 and 15. Many abundant tholin pyrolysis products satisfy this description. Tholins have a substantial absorption in the blue, responsible for their brown appearance, which is not due to S, and may be caused<sup>2</sup> by conjugated polyenes,  $\text{H}(\text{CH}=\text{CH})_n\text{H}$ , where  $n \geq 6$ . Our pyrolysis fragments<sup>3,4</sup> give good evidence for dienes in both tholins, and longer polyenes seem likely. If tholins are the principal constituents of the interstellar grains, the important conclusion follows that interstellar extinction is not merely due to scattering but must take account of substantial pure absorption as well ( $k \sim 10^3 \text{ cm}^2 \text{ g}^{-1}$  in the violet for UV tholins<sup>2</sup>).

The radii of particles ejected by stellar radiation pressure or stellar winds during the Hayashi or red giant stages of stellar evolution depend on the complex refractive index of the particles, but particles larger than a few  $\mu\text{m}$  are unlikely to be ejected. Thus the large tholin particles shown in Fig. 1 would not be ejected, but rather remain in circumstellar space, until degraded by sputtering or other processes into ejection-sized fragments. The smallest particles seen in Fig. 1*d* are, however,  $<1 \mu\text{m}$  in diameter and would thus be ejected, but they seem to have the size and at least some of the composition of interstellar grains.

From this general picture, we hypothesise<sup>10</sup> that the organic molecules detected by microwave line spectroscopy are chiefly spallation, spluttering or photodissociation products of tholins in interstellar grains, although UV and cosmic ray-induced positive ion-molecule reactions produce the spheric 1 interstellar organics in dense clouds. Typical lifetimes<sup>40</sup> of icy or dusty grains, unprotected by surrounding interstellar clouds, are  $10^8$ – $10^9$  years. The interstellar molecules should then resemble the pyrolyzate or MS cracking pattern of the tholins, or the vapourisation products of organics produced by low-temperature UV irradiation of condensates of abundant interstellar gases<sup>11</sup>. In Table 2 this comparison is performed for all identified interstellar molecules composed of H, C, O and N. For simplicity, we have neglected S compounds, but the array of such compounds in appropriate tholins<sup>3</sup> shows that no problems in interpretation are thereby posed. Every identified interstellar molecule either is produced directly in a relevant experiment (75%) or can be produced plausibly from tholin pyrolyzates (25%). Molecules with amino groups are in a special category, since they are degraded to nitriles during pyrolysis; however, the detection of amino acids in our UV tholins, with the very large quantum yield on hydrolysis of  $\sim 10^{-4}$ , shows<sup>1</sup> that amino groups or their precursors should be present in abundance. There is a tendency (Table 2), perhaps not yet statistically significant, for even C numbers and odd heavy atom numbers to be avoided in high molecular weight interstellar fragments; tholin pyrolyzates

do not show such a trend and it will be interesting to see whether microwave lines corresponding to 5, 7, 9 and 11 heavy atoms (such as, methacrylonitrile or toluene) will be found.

The investigation of cometary spectra has now yielded simple nitriles and aldehydes, molecules plausibly derived from cometary tholins formed in the solar nebula<sup>10</sup> and preserved in deep freeze for the intervening  $5 \times 10^9$  years. The similarities between cometary and interstellar molecules are sufficiently great that searches for a wide range of tholin pyrolyzates and their products seem desirable by remote spectroscopy, but particularly by *in situ* analytic chemistry in a cometary fly-through spacecraft mission. More generally, the tholin fragments identified in the laboratory<sup>3,4</sup> are plausible candidates for future studies of the interstellar medium by UV, visible, IR and microwave spectroscopy. Several molecules (HCN, CH<sub>3</sub>CN, HCHO, CH<sub>3</sub>CHO, C<sub>2</sub>H<sub>5</sub>OH) were found<sup>11,37</sup> in such laboratory experiments before their detection in the interstellar medium. The fact that molecules of some considerable complexity are predicted from the tholin argument suggests the need for comprehensive compilations of laboratory spectra of organic molecules, especially in the microwave region. The number of molecules is now so large for a finite microwave spectrum that statistically some molecules identified on the basis of one or two spectral lines may have been identified in error<sup>10</sup>.



**Fig. 4** Comparative IR spectra of *a*, UV tholin mix [Fig. 3 *f*]; *b*, Hoyle's<sup>29</sup> galactic 'best fit'; *c*, benzene-methanol extract of the Orgueil carbonaceous chondrite<sup>27,28</sup>; *d*, hot-water soluble, cold-water insoluble extract of the Mokoia carbonaceous chondrite.<sup>27,28</sup>

We believe the connection of this work with the origin and evolution of life on Earth to be indirect, although certainly interesting. The large scale production of complex organics in a preplanetary cloud, and the abundance of simpler organics in so inhospitable an environment as the present interstellar medium, surely imply that prebiological organic chemistry is easy, that much higher quantum yields and synthetic productivities should have applied on the primitive Earth. The presence of cyanoacetylene in the interstellar medium makes its presence, and the copolymerisation of biologically interesting molecules which it induces<sup>34</sup>, more likely on the primitive Earth. But the Earth's surface was probably molten in its early history, and organic

molecules are unlikely to have survived from the epoch preceding the formation of the Earth, although they should have accumulated thereafter. In any case the synthesis of organics on the primitive Earth seems to have been entirely adequate at least for our present level of understanding of the origin of life<sup>1,10,38</sup>. Surviving exogenous molecules are not needed. Likewise there seems to be no way for indigenous organisms to evolve—the heavy atom collision rate precludes more than about 50 interstellar generations in the age of the Galaxy and the organic reaction rates in cometary nuclei in the Oort cloud should be negligible—much less to arise in the interstellar medium<sup>10</sup>. If such organisms did exist<sup>29,39</sup>, their pathogenicity on Earth might be an open question, but organisms such as the influenza virus, with a molecular biology entirely typical of indigenous terrestrial organisms, are implausible in the extreme, as extraterrestrial pathogens. There may be a common organic chemistry on countless worlds as in the interstellar medium; but the stochastic nature of the evolutionary process makes it highly improbable that the biochemistry of extraterrestrial organisms will resemble very closely the biochemistry with which we are familiar on Earth.

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# Synthesis of rabbit $\beta$ -globin in cultured monkey kidney cells following infection with a SV40 $\beta$ -globin recombinant genome

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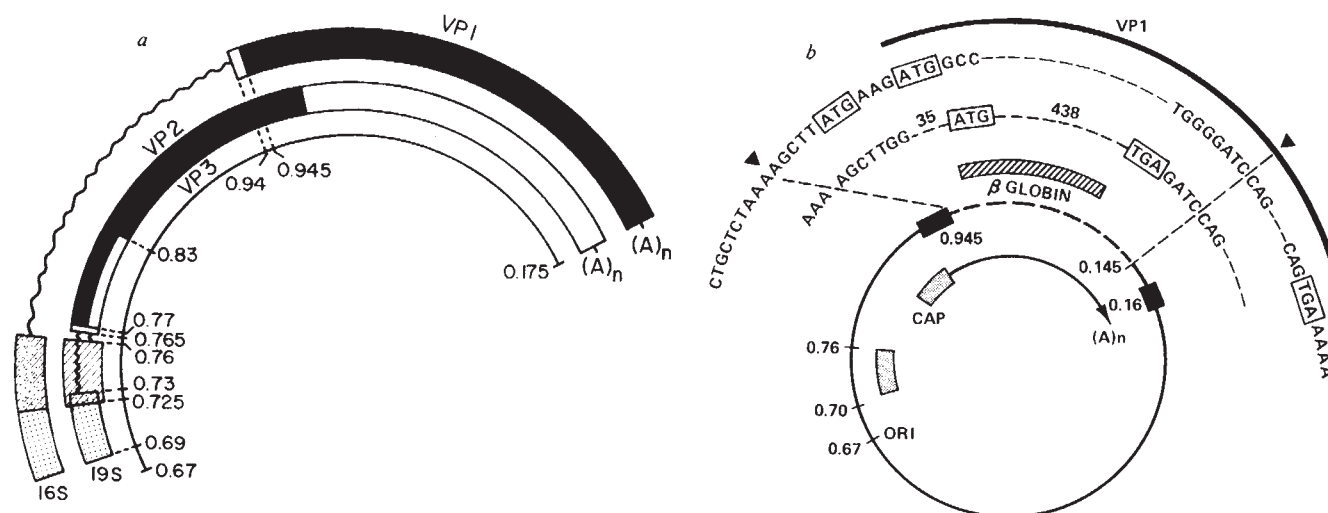
*Rabbit  $\beta$ -globin complementary DNA (cDNA) has been inserted into SV40 DNA in place of the gene coding for the virus' major capsid protein, VP1. The recombinant genome, SVGT5-Ra $\beta$ G, multiplies efficiently in CV1 monkey kidney cell cultures and is transcribed to yield cytoplasmic, polyadenylated hybrid mRNAs containing the  $\beta$ -globin coding sequence. Cells propagating SVGT5-Ra $\beta$ G produce substantial quantities of rabbit  $\beta$ -globin polypeptide.*

MAMMALIAN cells infected with SV40 virus carrying cloned eukaryotic genes provide one system in which the expression of eukaryotic genes can be studied<sup>1-3</sup>. SV40 is particularly attractive as a transducing vector for various reasons. The virus genome consists of a single, small, covalently closed circular DNA molecule, whose entire nucleotide sequence has recently been determined<sup>4,5</sup>; the viral DNA is obtainable in large quantities and relatively pure form; the genomic regions responsible for the various viral functions have been accurately located with

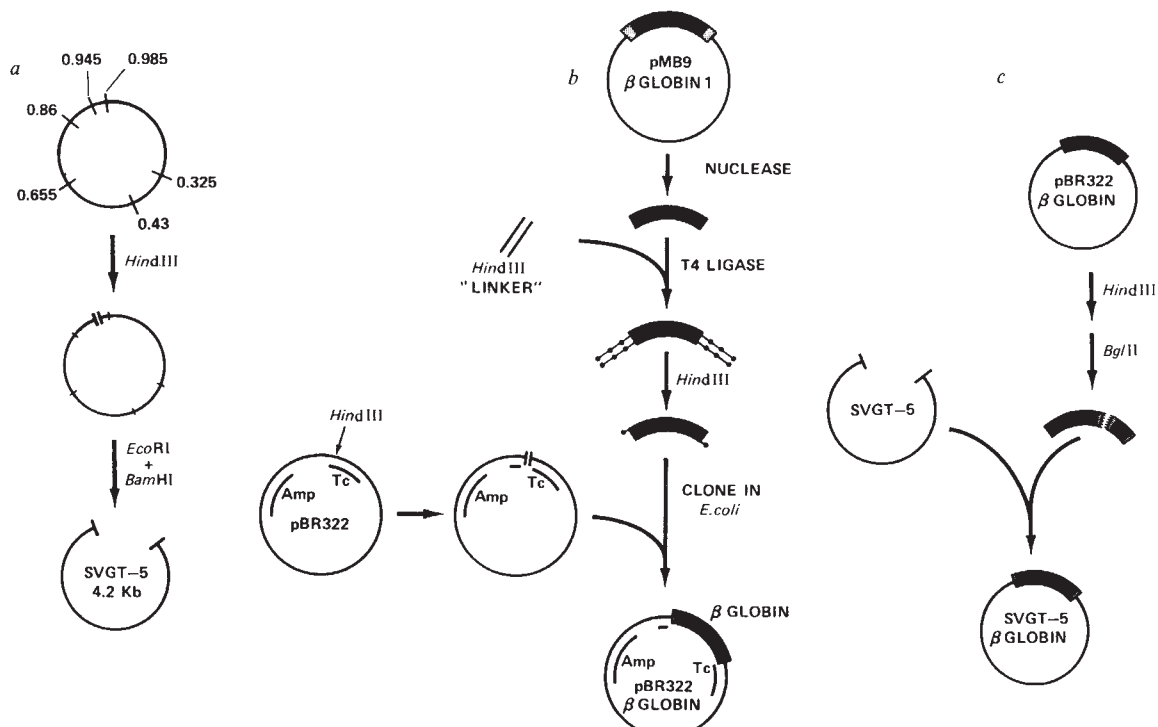
respect to a detailed physical map of the DNA<sup>4-7</sup>; the viral genome can multiply vegetatively or as an integral part of cellular chromosomes; and a wealth of information exists on the replication and expression of the viral genome and their regulation in different host cells.

To date a number of genes have been recombined *in vitro* with SV40 vectors and propagated in cultured monkey kidney cells; these include segments of  $\lambda$  phage DNA<sup>1,2</sup>, *Escherichia coli* DNA coding for tRNA<sup>Tyr</sup> (ref. 3), thymidine kinase (S. P. Goff and P. Berg, in preparation), guanine phosphoribosyl transferase (R. C. Mulligan and P. Berg, unpublished) and *Drosophila melanogaster* DNA containing the genes for H2A and H4 histones (H. Hofstetter and P. Berg, unpublished). Although the exogenous DNA segments are transcribed during infections with each of these recombinant genomes, discrete, mature functional mRNAs have not been detected.

We report here the construction and propagation of a recombinant genome in which virtually all of the coding sequence for the major capsid protein, VP1, located between map coordinates 0.945 and 0.145 (see Fig. 1a) has been replaced by a cDNA that specifies the entire amino acid sequence of rabbit  $\beta$ -globin<sup>8,9</sup>. Cells infected with the recombinant genome



**Fig. 1** a, The composite structure of SV40 late mRNAs. The map of SV40 DNA from the origin of DNA replication (0.67) to the end of the late region (0.175) is shown on the inner circle. The regions coding for the structure of VP1, VP2 and VP3 appear as shaded regions within bars that define the 'bodies' of the 19S and 16S mRNAs. The 'leader' segments are shown as bars spanning map coordinates 0.69–0.76. The cross-hatched and stippled regions of the 16S mRNA leader, for example, are intended as symbolic, rather than literal representations of more than one type of leader segment; one leader segment spans the region from 0.725 to 0.76 and another the region from 0.69 to 0.76. For the 19S mRNA species, one class of leaders spans map coordinates 0.69 to 0.73 and is joined to the body at 0.76; another class of leaders extends from 0.725 to 0.76. b, SV40 and rabbit  $\beta$ -globin cDNA nucleotide sequences relevant to the construction of SVGT5-Ra $\beta$ G. The origin of SV40 DNA replication at map position 0.67 is denoted as ori. SV40 16S late mRNA, with its 'capped' 5' leader sequence derived from 0.72 to 0.76 map units (stippled box), and 3' poly(A), is represented inside the circle. The region of the map coding for VP1 is represented by the heavy black line labelled VP1. The regions of SV40 DNA implicated in splicing and polyadenylation of the 16S mRNA are shown as black rectangles on the circular map. The dashed line extending leftward from map position 0.945 indicates the position of a *Hind*III endonuclease cleavage site in SV40 DNA (the outer sequence) and in pBR322- $\beta$ G2 DNA (the inner sequence). The dashed line extending rightward from map position 0.145 locates the unique *Bam*HI restriction site of SV40 DNA (outer sequence) and the *Bgl*II endonuclease cleavage site of pBR322- $\beta$ G2 (inner sequence). The nucleotide sequences between these dashed lines show the  $\beta$ -globin cDNA sequences inserted into SVGT5 and the SV40 sequences excised to construct SVGT5. The triplets enclosed in boxes show the initiation and termination signals for translation of VP1 and  $\beta$ -globin. The  $\beta$ -globin cDNA sequence in pBR322- $\beta$ G2 contains 35 nucleotides proximal to the initiator AUG in  $\beta$ -globin mRNA. The small hatched bar spans the region containing the initiation  $\beta$ -globin codon, the 438 nucleotides specifying the entire  $\beta$ -globin protein amino acid sequence, and the translation terminator codon.



**Fig. 2** Construction of SV40-RaβG recombinant genome. *a*, Construction of SVGT5 vector. Approximately 100 µg of SV40 DNA were incubated with 100 U of *Hind*III endonuclease (Boehringer) for 50 min at 30 °C in 10 mM Tris, pH 7.5, 5 mM MgCl<sub>2</sub>, 50 mM NaCl, 1 mM DTT. The partial digest was electrophoresed on a 0.8% agarose gel in Tris-borate buffer<sup>6</sup> to obtain full-length linear DNA molecules. The full-length linear DNA band was eluted electrophoretically from an excised gel slice, digested with an excess of *Bam*HI endonuclease (N.E. Biolabs) in 6 mM Tris, pH 7.5 containing 6 mM β-mercaptoethanol and 6 mM MgCl<sub>2</sub>; then, after adjusting the Tris to 100 mM, the mixture was digested with an excess of *Eco*RI endonuclease (N.E. Biolabs). The digest was electrophoresed on a 0.7% agarose gel and the largest band (4.2-kilobase) was excised and electroeluted. To remove residual agarose, the DNA was absorbed to a 0.5 ml DE-52 column equilibrated with TE (10 mM Tris, pH 7.5, 1 mM EDTA); after washing the column with TE the DNA was eluted with TE containing 1 M NaCl. *b*, Subcloning of β-globin cDNA. pBG1 DNA was incubated with 1,200 U of S<sub>1</sub> nuclease (Miles) for 30 min at 50 °C in buffer containing 30 mM sodium acetate (pH 4.5), 1 mM ZnSO<sub>4</sub>, 0.2 M NaCl and 45% (w/v) formamide<sup>17</sup>. The digestion was terminated by the addition of 10 mM EDTA; the DNA was precipitated with ethanol and electrophoresed in a 5% acrylamide gel in Tris-borate buffer for 3 h at 125 V. The ethidium bromide stained band containing the β-globin cDNA (0.5–0.58 kilobases) was excised and electroeluted. One µg of the β-globin cDNA was incubated with 3 µg *E. coli* polymerase I (provided by A. Kornberg) in 25 µl containing 60 mM Tris, pH 7.5, 8 mM MgCl<sub>2</sub>, 10 mM β-mercaptoethanol, 1 mM ATP, and 0.2 mM of each of the deoxynucleoside triphosphates for 10 min at 12 °C to convert 'ragged' ends into 'blunt' ends<sup>16</sup>. <sup>32</sup>P-labelled CCAAGCTTGG oligonucleotide (obtained from Collaborative Research) was added in a 50-fold molar excess relative to the cDNA termini and the reaction adjusted to contain 1 mM ATP. T4 DNA ligase (Bethesda Research Laboratory) (50 U ml<sup>-1</sup>) was added, the mixture (50 µl) incubated for 1 h at 20 °C and then heated at 80 °C for 5 min. After adjusting the salt concentration to 50 mM, *Hind*III endonuclease (15 units) was added and after 4 h at 37 °C, the digest was electrophoresed on a 5% acrylamide gel. The β-globin cDNA band was located by autoradiography, excised, and electroeluted. Approximately 0.7 µg of the recovered DNA was mixed with 1.2 µg of *Hind*III endonuclease-cleaved pBR322 DNA in 50 mM Tris buffer, pH 7.8, containing 10 mM MgCl<sub>2</sub>, 20 mM DTT, and 1 mM ATP (ligase buffer), 5 units of T4 DNA ligase were added and after an overnight incubation at 12 °C, suitable dilutions of the ligated mixture were introduced into HB101 (ref. 20). Transformants were selected on agar medium containing 20 µg ml<sup>-1</sup> ampicillin. Approximately 10<sup>4</sup> Amp<sup>r</sup> transformants were obtained per µg of ligated DNA. Amp<sup>r</sup> colonies were picked onto agar medium containing 50 µg ml<sup>-1</sup> tetracycline to identify recombinant plasmids with insertions at the *Hind*III restriction site<sup>21</sup>. Ten of 22 Tet<sup>r</sup> clones carried plasmids containing the β-globin insert. One isolate pBR322-βG2, served as the source of β-globin cDNA for insertion into SV40 DNA. *c*, Construction of SVGT5-RaβG. pBR322-βG2 DNA was cleaved sequentially with an excess of *Bgl*II and *Hind*III endonucleases in the buffers and conditions described above. The products were electrophoresed on a 4% acrylamide gel and the 485-base pair β-globin cDNA band was excised and electroeluted. Approximately 12 ng of the cDNA were incubated with 100 ng of SVGT5 DNA and T4 DNA ligase (25 U ml<sup>-1</sup>) in ligase buffer overnight at 12 °C. Dilutions of the ligated DNA and tsA58 DNA were used to transfect CV-1P cells as already described<sup>22</sup>. Fifty plaques were picked and (their viral DNAs) screened for the presence of β-globin cDNA sequences by a modification of the plaque hybridisation method of Villarreal and Berg<sup>23</sup>. A plate of confluent CV-1P cells (100 cm) was overlaid with 40 ml of agar medium and wells, 0.5 cm in diameter, were punched to expose small areas of the cell monolayer. An aliquot (10 µl) of each plaque suspension was added to a well, and after a 2 h incubation at 37 °C, the wells were filled with agar medium. After 3 d at 41 °C, the plate was overlaid with agar medium containing 0.01% Neutral Red and incubated overnight at 39 °C. The agar layer was removed and the cell monolayer transferred to a nitrocellulose disk<sup>23</sup>; the disk was annealed with [<sup>32</sup>P]-pBR322-βG2 DNA, washed and autoradiographed. Forty-six of the 50 plaques picked showed unmistakable evidence of hybridisation, thereby indicating the presence of β-globin cDNA sequences.

(SVGT5-RaβG) produce substantial quantities of discrete hybrid mRNAs that contain the β-globin coding sequence flanked by 5'-leader and 3'-terminal sequences characteristic of late SV40 mRNAs. The infected cells also synthesise rabbit β-globin polypeptide in quantities nearly equivalent to the amount of VP1 produced by the co-infecting helper virus.

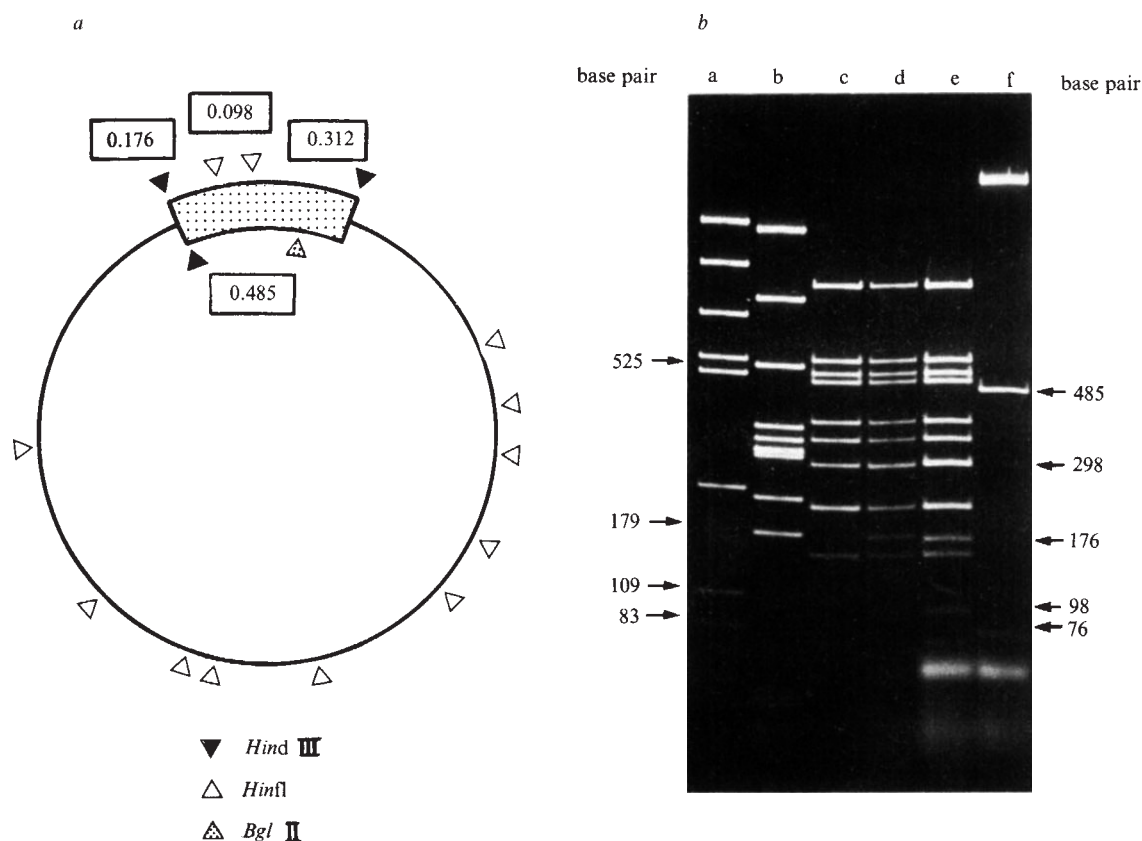
### Construction of SVGT5-RaβG

The mRNAs which code for SV40's three capsid proteins, VP1, VP2 and VP3 are composite structures, that is, the mRNAs contain nucleotide sequences transcribed from non-adjacent DNA segments<sup>10,11</sup>. Each mRNA contains a 5'-terminal segment (the leader) transcribed from the l-strand between map coordinates 0.69 and 0.76 (see Fig. 1a). Joined to the leader sequence, the VP2 and VP3 mRNAs (the 19S class) have a segment of RNA homologous to the l-strand between map coordinates 0.77 and 0.175 (the 19S body); the body of the VP1 mRNA (16S class) is transcribed from the l-strand of the DNA between map coordinates 0.94 and 0.175.

Deletion mutants have been obtained which lack the 3' boundary of the late region leader segments<sup>12</sup>. RNA transcribed from these mutants is not processed into mature 16 or 19S mRNA, presumably, because splicing cannot occur. Previously constructed SV40 vectors resemble these deletion mutants in that they have lost most or all sequences required for splicing. Very possibly the failure to obtain discrete mRNAs with the earlier recombinants stems, at least in part, from the consequent disruption of post-transcriptional splicing reactions. We have therefore sought to construct a SV40 vector which would retain all the regions implicated in transcriptional initiation and termination, splicing and polyadenylation of 16 and 19S mRNAs (see Fig. 1a).

Inspection of the SV40 restriction map suggested two restriction endonuclease-cleavage sites that could be used to generate such a vector. The first, a *Hind*III restriction site at map position 0.945, is six nucleotides proximal to the initiation codon for VP1 (refs 4,5) and about 50 nucleotides distal to the site at which the leader sequence is joined to the body of the VP1 mRNA<sup>13,14</sup> (see





**Fig. 3** Restriction analysis of pBR322-βG recombinants. *a*, The numbers in boxes are the expected sizes of restriction fragments, assuming the recovery of the complete cDNA sequence present in PβG1. The stippled region denotes the position of the β-globin cDNA insert and the various symbols indicate the particular restriction endonuclease cleavage sites. *b*, pBR322-βG DNAs were isolated as described by Katz *et al.*<sup>40</sup>, digested with an excess of the indicated enzymes and electrophoresed on a 3–7% gradient acrylamide gel<sup>6</sup>. The gel was stained with ethidium bromide (1 μg ml<sup>-1</sup>) and photographed under short wave ultraviolet light, using Polaroid 665 film and an orange filter. *a*, HindII endonuclease digested SV40 DNA. *b*, HaeIII endonuclease digested SV40 DNA. *c–e*, HindIII plus HindII endonuclease digested pBR322, pBR322-βG2, pBR322-βG3 DNAs, respectively. *f*, HindIII plus BglII digested pBR322-βG2 DNA.

Fig. 1*b*). The second, a *Bam*HI restriction site at map coordinate 0.145, is 50 nucleotides proximal to the termination codon for VP1 translation and 150 nucleotides before the poly(A) sequence at the 3'-end of VP1 mRNA<sup>4</sup>. The 4.18 kilobase DNA segment bounded by these two restriction sites (SVGT5) contains, in addition to the regions at which splicing and polyadenylation of late mRNAs occur, the origin of DNA replication and the entire early region; hence, it can be propagated by complementation with an appropriate *tsA* mutant<sup>15</sup>.

The rabbit β-globin cDNA sequence was selected as the transducible gene because it is small enough to be accommodated in SVGT5 and because the protein can be isolated and characterised readily. Furthermore, the cDNA had already been cloned in *E. coli* and shown to contain the complete coding sequence for β-globin, as well as additional 5' and 3'-terminal nucleotide sequences present in β-globin mRNA<sup>9</sup>. Our aim was to excise the β-globin cDNA segment from pMB9, modify and reclone it in another *E. coli* plasmid, pBR322 (ref. 16); in doing this we could obtain the β-globin cDNA segment with a *Hind*III cohesive end just proximal to β-globin's initiator codon (see Fig. 1*b*). Consequently, SVGT5 and the modified β-globin cDNA segment could be joined readily to form the desired recombinant genome.

### Construction, propagation and characterisation of SVGT5-RaβG genomes

To obtain SVGT5, wild-type SV40 DNA was partially digested with *Hind*III endonuclease to produce full-length linear molecules (Fig. 2*a*). Full-length linear DNAs were isolated by electrophoresis in agarose gel and digested with *Bam*HI and *Eco*RI

endonucleases. These cleavages, and the subsequent electrophoretic separation, yielded a SV40 DNA fragment of 4.18 kilobases which contained the nucleotide sequences between map coordinate 0.145, clockwise to the *Hind*III restriction site at map coordinate 0.945. The *Eco*RI endonuclease digestion was introduced to eliminate a potentially contaminating fragment of 4.28 kilobases formed by cleavages at map position 0.325 (a *Hind*III restriction site) and the *Bam*HI restriction site at 0.145.

The rabbit β-globin coding sequence was isolated from the PβG1 plasmid recombinant described by Maniatis *et al.*<sup>8</sup>. PβG1 was obtained by inserting a cDNA copy of purified rabbit β-globin mRNA at the *Eco*RI restriction site of the plasmid pMB9 using the poly(dA):(dT) joining method, and propagating the recombinant plasmid in *E. coli* (strain HB101)<sup>8</sup>. Nucleotide sequence analysis confirmed that PβG1 contained the entire β-globin coding sequence, all of the 3' and most (43 of 56 bases) of the 5'-non-coding sequences in β-globin mRNA<sup>9</sup>.

PβG1 DNA was digested with *S*<sub>1</sub> nuclease as described by Hofstetter *et al.*<sup>17</sup> to excise the β-globin cDNA segment from PβG1 (Fig. 2*b*). β-Globin cDNA fragments varying in size between 500–585 base pairs were recovered by electrophoresis in acrylamide gels and incubated with *E. coli* DNA polymerase I and the four deoxynucleoside triphosphates to convert 'ragged' into 'blunt' ends<sup>16</sup>. A decanucleotide 'linker', CCAAGCTTGG, containing the *Hind*III endonuclease recognition sequence, was phosphorylated at its 5'-end with polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP<sup>18</sup> and then joined to the ends of the β-globin cDNA with T4 ligase<sup>19</sup>; subsequent digestion with *Hind*III endonuclease generated *Hind*III cohesive ends.

The β-globin cDNA with its *Hind*III cohesive ends was ligated to *Hind*III endonuclease-cleaved pBR322 with T4 DNA ligase, and introduced into *E. coli* K12 (strain HB101)<sup>20</sup>. Trans-

formants were selected for ampicillin resistance and pBR322- $\beta$ G recombinants were screened for their sensitivity to high levels of tetracycline. (Cells containing pBR322 are resistant to ampicillin and tetracycline, but insertions at the *Hind*III restriction site eliminate resistance to high levels of tetracycline<sup>21</sup>.) Small cultures of each putative pBR322- $\beta$ G recombinant were grown, the plasmid DNA was isolated, cleaved with *Hind*III endonuclease and the products electrophoresed in agarose; 10 of 22 isolated plasmid DNAs yielded fragments 0.50 to 0.58 kilobases in addition to the linear pBR322 DNA (data not shown).

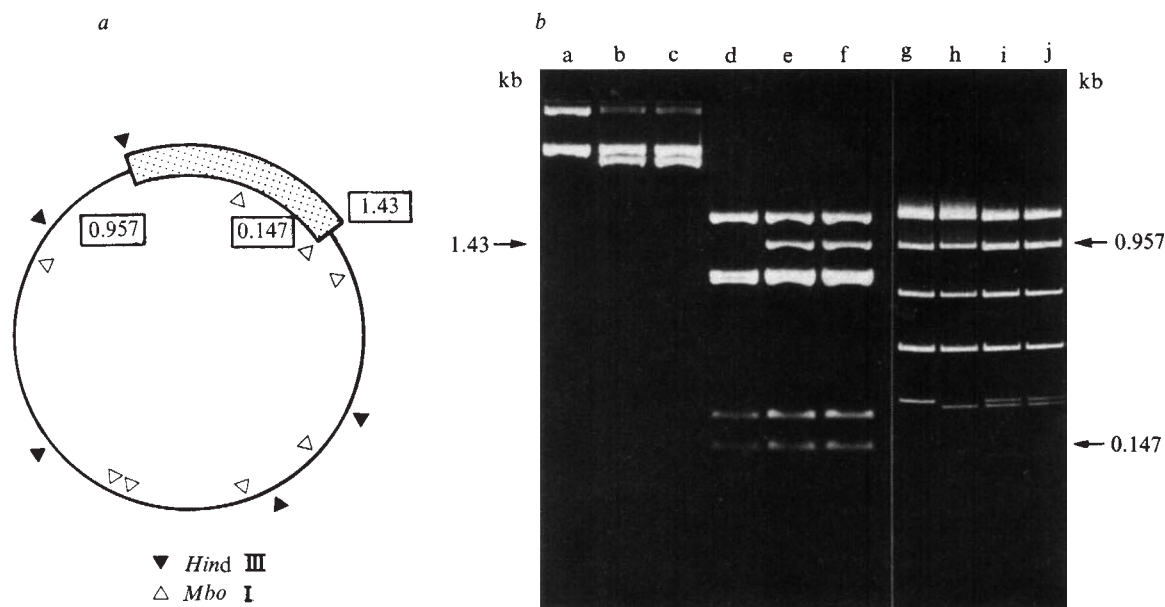
One of these, pBR322- $\beta$ G2, produced an insert fragment of about 570 base pairs following *Hind*III endonuclease digestion. Two fragments, 485 and 76 base pairs long were detected by digesting this plasmid with *Hind*III and *Bgl*II endonucleases (Fig. 3 track f). These findings show that the expected *Hind*III joins are intact, that they are separated from each other by nearly the expected length of the  $\beta$ -globin cDNA segment and that the single internal *Bgl*II restriction site in the  $\beta$ -globin cDNA is present. These conclusions are reinforced by the double digestions of pBR322- $\beta$ G2 and pBR322- $\beta$ G3 with *Hind*III and *Hinf*I endonucleases (Fig. 3 tracks d and e). Fragments corresponding to the expected 176 base pair fragment (derived from the 5'-end of the  $\beta$ -globin coding sequence) and 98 base pair fragment (produced from an internal sequence in the cDNA) were readily apparent in the electropherogram. The predicted 312-base pair fragment, generated from the 3'-end of the globin coding sequence, is actually 298 base pairs and is obscured by a fragment of the same size derived from the vector DNA. This was deduced by inspection of the original film in which the staining of the 298-base pair band was more intense than the band migrating behind it. Determination of the nucleotide sequences<sup>18</sup> at the two *Hind*III join sites in pBR322- $\beta$ G2 further supports the restriction enzyme analysis and establishes that 5 nucleotides at the 5'-end, 15 nucleotides at the 3'-end and all of the dA:dT sequences of the originally cloned  $\beta$ -globin cDNA are absent in the  $\beta$ -globin cDNA segment recloned in pBR322 (data not shown).

The  $\beta$ -globin cDNA segment of pBR322- $\beta$ G2 was excised by sequential digestion with *Hind*III and *Bgl*II endonucleases (Fig. 2c). This yielded a fragment having the codon for initiating

translation of  $\beta$ -globin 37 nucleotides beyond the *Hind*III endonuclease-generated cohesive end and the translation termination codon just proximal to the *Bgl*II endonuclease-generated cohesive end. Even though the *Bam*HI (GGATCC) and *Bgl*II (AGATCT) endonuclease recognition sites differ, the cohesive ends generated by the two endonucleases are identical (GATC). Accordingly, the 0.485-kilobase  $\beta$ -globin cDNA fragment could be ligated to SVGT5 with T4 ligase<sup>24</sup> (Fig. 2c). To propagate the SVGT5- $\beta$ G2 genome, CV1-P cells were transfected with a mixture of the ligated DNA and *tsA58* DNA<sup>22</sup>. *tsA58* is a thermosensitive mutant of the SV40 early function and can not multiply at 41 °C (ref. 15). About  $2.5 \times 10^4$  plaques were obtained per microgram of ligated DNA.

These plaques were screened for virus containing the  $\beta$ -globin cDNA using a modification of the *in situ* plaque hybridisation technique<sup>23</sup> (see legend to Fig. 2c). Forty-six of fifty plaques examined contained DNA that hybridised specifically to the  $\beta$ -globin cDNA. Recombinant virus from independent clones were purified by plaque isolation and high-titre virus stocks and purified viral DNA were prepared in CV1 cells. Judging from the relative yields of the two DNAs from such mixed infections, we estimate that the recombinant and helper genomes multiply equally well.

As expected from its somewhat smaller size (4.7 compared with 5.2 kilobases), SVGT5- $\beta$ G DNA is separable from the helper *tsA58* DNA by electrophoresis in agarose (Fig. 4, tracks a-c). Restriction endonuclease digestions and electrophoresis of the products helped to confirm the structure of the hybrid DNA. The locations of the *Hind*III and *Mbo*I restriction sites in SVGT5 and the  $\beta$ -globin cDNA sequence are indicated in Fig. 4a. Correct joining of the  $\beta$ -globin cDNA to SVGT5 regenerates the *Hind*III restriction site at map position 0.945. Therefore, digestion of SVGT5- $\beta$ G DNA with *Hind*III endonuclease should generate a fragment of 1.43 kilobases from cleavages at the *Hind*III join sequence and the *Hind*III restriction site at map position 0.325 in the vector; the 1.43-kilobase fragment is produced in the *Hind*III endonuclease digests of two independent recombinants, SVGT5- $\beta$ G 10A and SVGT5- $\beta$ G 12A (Fig. 4, tracks e and f), but is absent from a comparable digest of SV40 DNA (track d). After transfer of the DNA in the gel to a nitrocellulose sheet<sup>25</sup> and hybridisation with [<sup>32</sup>P]  $\beta$ G1



**Fig. 4** Restriction analysis of SVGT-Ra $\beta$ G. *a*, The stippled region denotes the  $\beta$ -globin cDNA sequence and the numbers enclosed in boxes are the expected fragment sizes based on the sequence of the  $\beta$ -globin cDNA in pBR322- $\beta$ G2 and the known locations of the *Hind*III and *Mbo*I restriction sites. *b*, Viral DNA was extracted by Hirt's method<sup>27</sup> from CV1 cells that had been infected with plaque-purified SVGT5- $\beta$ G and *tsA58* virus (M.O.I. 0.005 PFU per cell). The DNA was centrifuged to equilibrium in ethidium bromide-caesium chloride gradients<sup>28</sup>; aliquots were then digested with the appropriate enzymes and electrophoresed on either 0.8% agarose gels (tracks a-f) or on 3-7% gradient acrylamide gels (tracks g-j). Tracks a-c: undigested SV40, SVGT5- $\beta$ G10A and SVGT5- $\beta$ G12A DNAs. Tracks d-f: the same three DNAs digested with *Hind*III. Tracks g-h: *Mbo*I endonuclease digests of SV40, *tsA58*, SVGT5- $\beta$ G10A and SVGT5- $\beta$ G12A DNAs.



DNA, only the 1.43-kilobase fragment was labelled (data not shown); hence, all of the  $\beta$ -globin cDNA sequence is contained within that fragment.

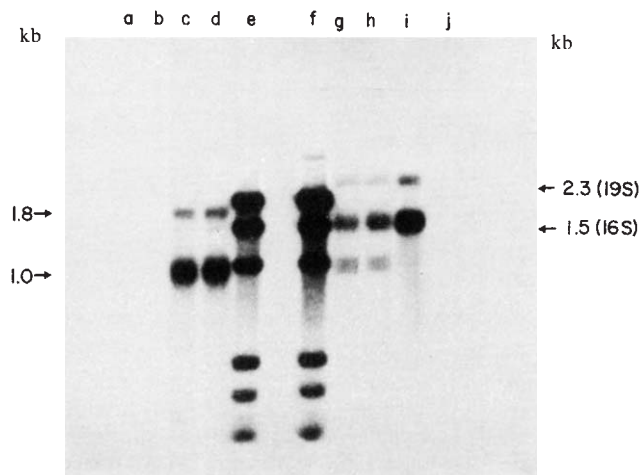
Ligation of *Bam*HI and *Bgl*II endonuclease-created cohesive ends should generate a sequence which is resistant to cleavage by these two enzymes but sensitive to *Mbo*I endonuclease (recognition sequence NGATCN). Indeed, *Mbo*I endonuclease digestion of SVGT5- $\beta$ G 10A and SVGT5- $\beta$ G 12A DNAs produced the expected array of fragments (Fig. 4b). The 0.957 fragment, which spans the *Hind*III join site, comigrates with one of the vector DNA fragments (compare tracks i and j with g and h); the 0.147-kilobase fragment, formed from cleavages at a *Mbo*I restriction site within the  $\beta$ -globin cDNA and the *Mbo*I site formed by the join of the *Bgl*II and *Bam*HI cohesive ends is produced from both recombinants (tracks i and j) but is absent from SV40 DNAs (tracks g and h). Note that the 0.240 fragment produced from the SV40 DNA used to make the vector (track g) is slightly larger than the corresponding fragment cleaved from the *tsA58* helper DNA (track h). The mixture of recombinant and helper DNA yielded both fragments in about equal amounts indicating that here too the recombinant and helper genomes were equally represented in the infection from which these DNAs were isolated.

Although not shown, 8 of 14 other independent clones of SVGT5- $\beta$ G gave restriction digests comparable to the two documented here. These data indicate that the  $\beta$ -globin cDNA cloned in pBR322 has been introduced into SVGT5 in the expected way.

## Formation of SV40-Ra $\beta$ -globin hybrid mRNAs

Considering the structure of SVGT5- $\beta$ G and present ideas about SV40 late mRNA formation, two cytoplasmic SV40- $\beta$ -globin hybrid mRNAs should be formed following infection with SVGT5- $\beta$ G. These should be about 0.5 kilobases smaller than the normal SV40 19S and 16S late mRNAs. To test these expectations, CV1 cells were infected with *tsA58* and each of two SVGT5- $\beta$ G recombinant viruses (MOI 10–50 PFU per cell) at 37 °C and, after 48 h, cytoplasmic, poly(A)-containing RNA (freed from DNA) was isolated (see legend to Fig. 5); similar RNA preparations were isolated from SV40 and mock-infected cultures. Each RNA preparation was denatured with glyoxal<sup>26</sup>, electrophoresed on 1.5% agarose gel and transferred to diazotised benzyloxymethyl paper<sup>12,30</sup>. The imprints were annealed with <sup>32</sup>P-DNA to reveal the positions of the separated RNAs (Fig. 5).

With [<sup>32</sup>P] p $\beta$ G1 DNA as hybridisation probe, two discrete  $\beta$ -globin mRNA species are detectable in the RNA from cells infected with either recombinant (Fig. 5, tracks c and d). These RNAs are missing in the RNA from mock or SV40-infected cells (tracks a and b). With DNA fragments of known size as length standards (tracks e and f) we estimate the sizes of these mRNAs as 1.8 and 1 kilobases. When duplicate tracks were hybridised with [<sup>32</sup>P]-SV40 DNA containing the late mRNA leader sequences, two labelled bands are visible in the SV40-infected cell RNA (track i), but not in the RNA from mock-infected cells (track j). The faster migrating, more heavily labelled band is the 16S mRNA (1.5 kilobase) and the slower, less heavily labelled band the 19S mRNA (2.3 kilobase). There are four labelled bands when RNA from cells infected with the two SVGT5- $\beta$ G recombinants is hybridised with the labelled SV40 leader probe (tracks g and h). Two (the 1.5 and 2.3-kilobase species) are the 16S and 19S mRNAs produced by the *tsA58* helper genome. Another has the mobility of the 1-kilobase RNA species that hybridised to the globin cDNA probe. The fourth, which is only barely discernable in this exposure, has a mobility very close to that of the 1.8-kilobase RNA detected with the  $\beta$ -globin cDNA probe. Thus the 1 and 1.8-kilobase polyadenylated RNAs contain the  $\beta$ -globin cDNA sequence and the leader segment characteristic of SV40 late mRNAs. Comparing the labelling intensities of the 1-kilobase  $\beta$ -globin mRNA and the 1.5-



**Fig. 5** Analysis of mRNAs made in cells infected with SVGT5- $\beta$ G. Cytoplasmic poly(A)-containing RNA was isolated from CV1 cells 48 h after infection with a mixture of SVGT5- $\beta$ G and *tsA58* virus (>10 PFU per cell) as follows. Cells were washed twice with ice-cold Tris-buffered saline and once with a cold solution containing 10 mM Tris, pH 7.5, 5 mM MgCl<sub>2</sub>, 10 mM NaCl. The cell monolayer, in the latter buffer containing 0.1% NP40 and 0.05% deoxycholate (1 ml per plate), was scraped off the plate with a rubber policeman and centrifuged for 2 min at 1,500 r.p.m. to pellet the nuclei. The supernatant was mixed with an equal volume of solution containing 0.3 M NaCl, 0.1 M Tris, pH 8.0, 10 mM EDTA, and 0.15% SDS. Cell aggregates were dispersed by pipetting and the suspension was extracted two times with an equal volume of water-saturated phenol-chloroform (1:1). After ethanol precipitation, the nucleic acid was digested with iodoacetate-treated DNase<sup>29</sup> (Worthington) (20  $\mu$ g per mg nucleic acid), at room temperature for 45 min in a small volume of 20 mM Tris, pH 7.5, 10 mM NaCl, and 10 mM MgCl<sub>2</sub>. The mixture was extracted twice with phenol-chloroform and passed over poly(U)-Sephacrose<sup>41</sup> to isolate poly(A)-containing RNA. Aliquots of the poly(A)-containing RNA were denatured with glyoxal<sup>26</sup> and electrophoresed in a 1.5% agarose gel in 10 mM sodium phosphate pH 7.0. The fractionated RNA was then transferred to diazotised benzyloxymethyl paper<sup>30</sup> as described by Villarreal, White and Berg<sup>12</sup>. The paper was annealed with about  $4 \times 10^6$  c.p.m. of the indicated <sup>32</sup>P-nick-translated DNA probe for 24 h, then washed<sup>12</sup> and autoradiographed for 2 d. <sup>32</sup>P-labelled pBR322- $\beta$ G2 DNA fragment (from map position 0.67–0.76, produced by cleavages with *Bgl*II and *Hpa*I endonucleases) was the hybridisation probe for tracks g–j. Tracks e and f contained a *Hind*III digest of [<sup>32</sup>P] SV40 DNA as size standards. The lengths of the fragments (kilobases) in decreasing order are: 1.96, 1.54, 1.07, 0.369, 0.240. Tracks a, j; RNA from non-infected cells; b, i SV40 RNA; c, h SVGT5- $\beta$ G10A RNA; d, g SVGT5- $\beta$ G12A RNA.

kilobase SV40 mRNA (in Figs, tracks g and h) and taking account of the ratio of recombinant to helper DNA in these infected cultures (0.3 recombinant), we surmise that the steady state levels of the two mRNAs are within a factor of two of each other.

## Formation of $\beta$ -globin

One goal of our experiment was to determine whether replacing the VP1 coding sequence with the  $\beta$ -globin sequence would permit the formation of a discrete, cytoplasmic SV40- $\beta$ -globin hybrid mRNA. An additional goal was to determine whether such SV40- $\beta$ -globin hybrid mRNAs could be translated *in vivo* to produce  $\beta$ -globin. CV1 cells were infected with *tsA58* and either of the two SVGT5- $\beta$ G recombinants (MOI 10–50 PFU per cell) and, after 48 h (37 °C), the cultures were labelled with <sup>3</sup>H-leucine and <sup>3</sup>H-valine for 30 min; SV40 and mock-infected cultures were labelled in a comparable way. An extract of each culture was prepared by sonication and treatment with NP40-deoxycholate; aliquots were electrophoresed on a SDS 15–20% gradient acrylamide gel (see legend to Fig. 6). The autoradiograms of each electrophoresed extract had a complex array of labelled protein bands (Fig. 6 tracks a–d). But the patterns from the SVGT- $\beta$ G infected cell extracts (tracks c and d) contain a protein band migrating at the position of added rabbit  $\beta$ -globin; this protein band is not discernable in either the SV40 or mock-infected cell extracts (tracks a and b). The formation of a  $\beta$ -globin-like protein in cells infected with SVGT5- $\beta$ G is supported by the fact that a protein with the same mobility as authentic rabbit  $\beta$ -globin can be recovered from complexes

formed by incubating the cell extracts with purified goat anti-rabbit  $\beta$ -globin immunoglobulin (kindly provided by S. Boyer, Johns Hopkins) and heat killed, formalin-fixed *Staphylococcus aureus* cells<sup>33</sup>; the electrophoretic pattern of the labelled proteins eluted with SDS and urea from such complexes are shown in tracks e-h.  $\beta$ -globin bands are seen in the extracts from cells infected with the recombinant (tracks g and h), but not in extracts from uninfected and SV40-infected cells (tracks e and f). If the extracts from SVGT5- $\beta$ G infected cells are mixed with non-immune goat serum (tracks i and j) or anti-SV40 VP1 serum (track k), before adding the *S. aureus* cells, no  $\beta$ -globin is detectable in the electrophoresed samples.

In a preliminary experiment (carried out in collaboration with T. Hunter at the Salk Institute) the putative  $\beta$ -globin protein obtained from cells infected with SVGT5- $\beta$ G was immunoprecipitated with the immune serum and *S. aureus* cells mentioned above, eluted with SDS, digested with trypsin and analysed by the two-dimensional electrophoretic-chromatographic procedure<sup>36</sup>. The tryptic peptide maps of the putative  $\beta$ -globin and labelled rabbit  $\beta$ -globin synthesised *in vitro*<sup>36</sup> were strikingly similar; at least 8 of 10 peptide spots in the two maps matched (data not shown). Further experiments to characterise the  $\beta$ -globin, particularly the amino and carboxy terminal amino acid sequences, are in progress.

In experiments to be published later we have compared the amounts of  $\beta$ -globin and VP1 synthesised in cells infected with SVGT5- $\beta$ G and the helper virus tsA58. <sup>35</sup>S-methionine labelled infected cell extracts were mixed with either anti- $\beta$ -globin or anti-SV40 VP1 serum, both immune complexes were adsorbed to *S. aureus* cells, washed, eluted with SDS, electrophoresed in SDS-polyacrylamide gel and autoradiographed. The amount of <sup>35</sup>S-label in  $\beta$ -globin and VP1 was quantitated by densitometry of their respective bands in the autoradiograms. Taking into account the difference in the number of methionine residues per mole of  $\beta$ -globin and VP1 (1:9) and the ratio of SVGT5- $\beta$ G to tsA58 genomes during the period of protein labelling (1:3), we estimate that  $\beta$ -globin and VP1 are synthesised at nearly equal rates.

## Discussion

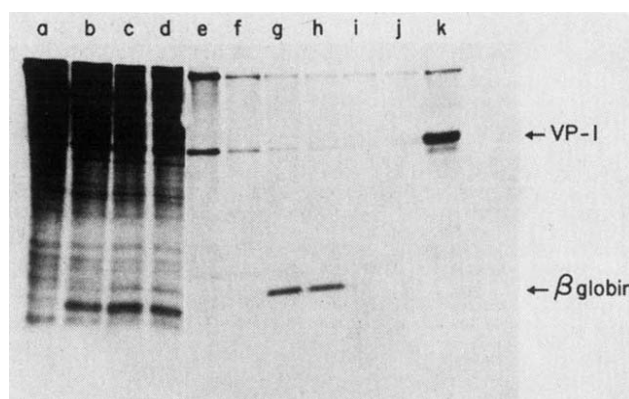
These experiments establish that an exogenous gene, in this case the coding segment for rabbit  $\beta$ -globin, can be recombined with SV40 DNA *in vitro* to yield a hybrid genome that expresses the incorporated gene. The principal conceptual innovation is the decision to leave intact the regions in the vector implicated in SV40 late mRNA processing: specifically, the late mRNA leader sequence, the sequence to which the leader is spliced during maturation of late mRNAs, and the region in which late transcripts terminate and are polyadenylated (see Fig. 1a and b). In the recombinant genome, virtually all of the coding sequence of SV40's major capsid protein VP1 has been replaced by a  $\beta$ -globin cDNA.  $\beta$ -globin's initiator codon, and presumably its ribosome-binding sequence, replace the corresponding initiation sequence of VP1; furthermore,  $\beta$ -globin's translation terminator codon is proximal to the VP1 termination codon remaining in the vector (Fig. 1b). This strategy was adopted so that the  $\beta$ -globin coding sequence would be incorporated into mature SV40- $\beta$ -globin hybrid mRNAs and be translated.

The success of the experiment clearly demonstrates that processing of both the 16 and 19S late mRNAs does not require nucleotide sequences within the VP1 coding region: analogous (smaller) mRNAs with poly(A) and leader sequences are formed efficiently when most of the VP1 coding sequence is replaced by the  $\beta$ -globin cDNA. Detailed studies are needed to compare the nucleotide sequences of wild-type 16 and 19S mRNA leaders with SVGT5- $\beta$ G's corresponding  $\beta$ -globin mRNA leader sequences; it may also be interesting to compare the relative rates of the various splicing reactions of normal and recombinant RNAs. Another intriguing question is whether the monkey cells can excise the two intervening sequences present in the rabbit  $\beta$ -globin genomic DNA segment (Maniatis, T. *et al.*, in preparation), a question which can be examined with

recombinants in which the rabbit  $\beta$ -globin genomic DNA sequence has been inserted into analogous SVGT vectors.

VP1 is believed to be translated exclusively from the 16S mRNA although the protein's coding sequence is also contained within the 19S mRNA<sup>37</sup>. Therefore,  $\beta$ -globin is probably translated from the 1-kilobase SV40- $\beta$ -globin hybrid mRNA (the 16S mRNA analogue) and not the 1.8-kilobase hybrid mRNA (the 19S mRNA analogue). Conceivably, however,  $\beta$ -globin may be translated from the 19S mRNA analogue as well, either because the altered nucleotide sequence of the large hybrid mRNA significantly affects its secondary structure or because the ribosome binding site for  $\beta$ -globin is functionally independent of its position within a message. Experiments are in progress to insert the  $\beta$ -globin coding sequence at several different locations in the late and early regions of SV40 DNA, making it possible to assess the effect of varying the distance of the  $\beta$ -globin coding sequence from the 5'-end of the mRNA, the role of neighbouring sequences and any regulatory influence on the efficiency of expression—transcription, processing and translation—of the  $\beta$ -globin 'gene'.

Of particular interest is whether other genes can be expressed if they are inserted into appropriate SV40 DNA vectors in a similar way. Hofstetter and Berg (unpublished results) have



**Fig. 6** Production of rabbit  $\beta$ -globin in cells infected with SVGT5-Ra $\beta$ G. Subconfluent CV1 cells were infected with a mixture of SVT5- $\beta$ G and tsA58 (>10 PFU per cell) for 48 h at 37 °C in a medium containing 4% fetal bovine serum. The cells were washed once with Tris-buffered saline and once with medium lacking leucine and valine. Cells were labelled for 30 min at 37 °C with 200  $\mu$ Ci each of <sup>3</sup>H-leucine and <sup>3</sup>H-valine (Amersham-Searle) in 1 ml of medium containing 4% dialysed calf serum but lacking leucine and valine. The labelled cells were washed twice in ice-cold Tris-buffered saline, scraped off the plate in Tris-buffered saline containing 10 mM DTT and 250  $\mu$ g ml<sup>-1</sup> PMSF (added fresh) (1 ml per 100 cm plate)<sup>31</sup> and then sonicated for 2 min in a water-cooled Heat Systems sonicator cup horn (maximum setting). Extracts were made 0.1% in NP40 and 0.05% in deoxycholate, incubated on ice for 10 min, centrifuged at 18,000 r.p.m. for 30 min, and the supernatants stored in aliquots at -80 °C. Aliquots of each extract were reacted with specific antisera or antibody proteins according to the method of Padgett *et al.*<sup>32</sup>. Extracts of approximately 2.5  $\times$  10<sup>6</sup> cells were preincubated with 50  $\mu$ l of a 10% suspension of heat-killed formalin-fixed *Staphylococcus aureus* Cowen 1 bacteria<sup>33</sup> in 25 mM Tris, pH 7.4, 10 mM EDTA, 0.35 M NaCl, and 0.15% Triton X-100 (TENT buffer) for 15 min on ice. After removal of the bacteria by centrifugation, purified rabbit  $\beta$ -globin (1  $\mu$ g) and an excess of either purified goat anti-rabbit  $\beta$ -globin immunoglobulin (provided by S. Boyer, Johns Hopkins), normal goat serum (Microbiological Associates), or rabbit anti-VP1 serum (provided by J. Reiser, Stanford) was added and the extracts incubated overnight at 4 °C. Then 50  $\mu$ l of *S. aureus* suspension (as described above) were added, the mixtures were incubated for 10 min at 4 °C, and centrifuged for 2 min at 8,000 r.p.m. The bacterial pellet was washed four times with TENT buffer and the immune complexes eluted from the bacteria for 1 h in a solution containing 10 mM Tris pH 7.4, 1% SDS, and 8 M urea. Electrophoresis of cell extracts or of immune complexes prepared from those extracts was for 3 h at 125 V in a 15–20% gradient acrylamide-SDS gel prepared according to the method of Laemmli<sup>34</sup>. The gel was stained and fluorographed<sup>35</sup> (3-day exposure shown). Tracks a-d are untreated cell extracts; e-h are immune complexes from extracts reacted with goat-anti-rabbit  $\beta$ -globin immunoglobulin; a, e, uninfected cell extract; b, f, SV40-infected cell extract; c, g, SVGT5- $\beta$ G10A-infected cell extract; d, h, SVGT5- $\beta$ G12A-infected cell extract. Tracks i, j: immune complexes prepared from SVGT5- $\beta$ G10A (i) or SVGT5- $\beta$ G12A (j) infected cell extract reacted with normal goat serum; k, immune complex prepared from SVGT5- $\beta$ G10A-infected cell extract reacted with rabbit anti-VP1 serum.



inserted a *Drosophila melanogaster* (Dm) DNA segment containing the gene for histone H2B into SV40 DNA (between map positions 0.825 and 0.145) and observed the formation of both a cytoplasmic, polyadenylated SV40-Dm H2B hybrid mRNA and H2B protein during multiplication of the recombinant in monkey cells. Experiments are in progress to examine the expression of a variety of prokaryotic, lower eukaryotic and mammalian genes in mammalian cells using this approach.

SVGT5-Ra $\beta$ G may prove invaluable for testing the effects of mutational alterations on the regulation of SV40 transcription. Deletions could be introduced into those portions of the vector DNA that are implicated in viral mRNA formation and their consequences determined, even in the presence of helper genomes, by monitoring  $\beta$ -globin mRNA and protein synthesis. This approach will be particularly advantageous where the mutational changes either prevent or have no effect on the transcription of particular messages. More generally, this system may be used in conjunction with new methods for site-directed mutagenesis<sup>38,39</sup> to 'synthesise' altered eukaryotic mRNA structures at the DNA level, and to examine their function *in vivo*.

SVGT5-Ra $\beta$ G should be able to transform a variety of mammalian cells, such as mouse, rat and human. Since integrated SV40 genomes do not express the late region, SVGT5- $\beta$ G transformed cells are not likely to make  $\beta$ -globin mRNAs or  $\beta$ -globin. However, other vectors, which permit transcription of the exogenous gene under early control are

being explored for their ability to promote expression of the integrated genes.

$\beta$ -Globin made during infection of CV1 cells by SVGT5- $\beta$ G is not stable; following a 30-min labelling period most of the newly synthesised  $\beta$ -globin disappears during a 60 min chase (data not included). Several explanations come to mind: the newly synthesised polypeptide is an altered form of  $\beta$ -globin; factors in the monkey cell cultures actively promote breakdown of  $\beta$ -globin chains; cultured monkey kidney cells lack factors normally present in erythroid cells that stabilise  $\beta$ -globin. Our agenda includes experiments to characterise further the structure of rabbit  $\beta$ -globin synthesised in monkey cells and to determine if the  $\beta$ -globin can be stabilised, perhaps by the addition of haemin to the culture medium or the concomitant production of rabbit  $\alpha$ -globin by co-infection with a SVGTaG recombinant genome.

We thank Dr T. Maniatis for providing the *E. coli* culture harbouring the  $\beta$ -globin recombinant plasmid p $\beta$ G1. All experiments involving the construction and propagation of recombinant genomes were performed according to the stipulations in the existing NIH guidelines: P2 for p $\beta$ G1 and pBR322- $\beta$ G and P3 for SVGT5- $\beta$ G. This research was supported by research grants NIH GM 13235-13 and NCI CA 15513-05. Richard C. Mulligan is a NSF Fellow and Bruce H. Howard is an Investigator in the USPHS.

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## letters

### Interstellar reddening and distance of Nova Cygni 1978

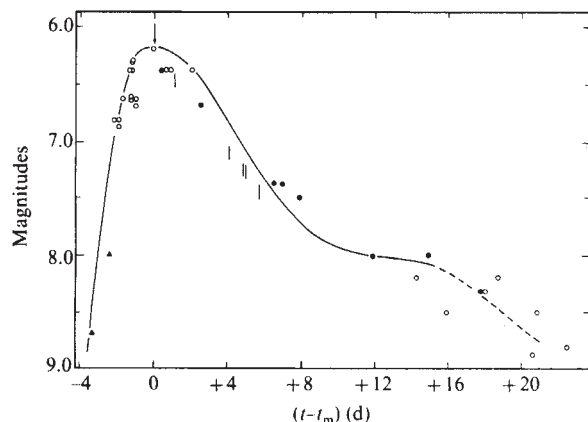
THE interstellar feature of KI( $\lambda$ 7699 Å) has been observed in the direction of Nova Cygni 1978 with an equivalent width of  $W_\lambda = 116$  mÅ and has been used here to deduce a colour excess of  $E(B - V) = 0.38 \pm 0.08$  mag. The distance-reddening law to the nova is derived from a sample of stars in the nearby field surrounding the nova. The nova seems to be at a distance of  $r = 3.3 \pm 0.6$  kpc with a corresponding absolute visual magnitude at maximum light of  $M_{V_0} = -7.5 \pm 0.5$  mag. Nova Cygni 1978 is classified as a fast galactic nova on the basis of its absolute magnitude at maximum light and the rate of decline towards minimum light.

A composite light curve for Nova Cygni 1978 is shown in Fig. 1; the data are a mixture of visual, photographic (prediscovery)

and photoelectric measurements<sup>1-3</sup>. A polynomial fit to the data yields a time of maximum light of  $t_m = 12.20 \pm 0.04$  September 1978 (UT) when the nova attained a brightness of  $m_v = 6.2 \pm 0.1$  mag. Near maximum light and thereafter, high resolution spectra (220 mÅ) were obtained of the nova using the 2.7-m telescope of the McDonald Observatory with a self-scanned Reticon 1024B detector<sup>4</sup> and the coude spectrograph.

In addition to monitoring various emission features, including H $\alpha$ , H $\beta$ , OI( $\lambda$ 8446 Å), and HeI( $\lambda$ 10830 Å), spectra were also obtained of the NaI( $\lambda$ 5890 Å) and KI( $\lambda$ 7699 Å) interstellar lines.

The interstellar NaI lines are very strong, having equivalent widths  $\sim 550$  mÅ; NaI lines arising from the nova are also seen displaced by 626 km s<sup>-1</sup> to the blue. The KI line, shown in Fig. 2, is weaker and seems to contain two unresolved components. From the measured equivalent width of the KI line ( $W_\lambda = 116$  mÅ) a column density of  $N(KI) = 9.3 \times 10^{11}$  cm<sup>-2</sup> is



**Fig. 1** Composite light curve for Nova Cygni 1978.  $\blacktriangle$ , Predisccovery photographic values;  $\bullet$ , photoelectric observations;  $\circ$ , visual observations. Time of maximum light,  $t_m$ , (arrow) and the epochs of the spectroscopic data (vertical lines) are indicated. Solid curve approximates behaviour of nova during rise to maximum and early decline.

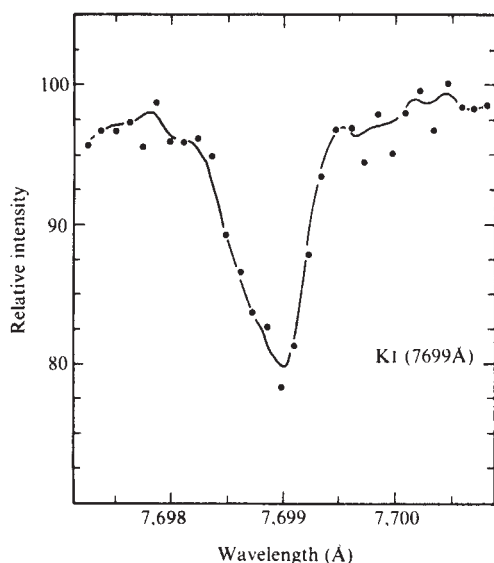
deduced. This column density can be converted in the  $E(B - V)$  colour excess using the mean relationship<sup>5</sup>

$$N(KI) = 6.4 \times 10^{12} \cdot E(B - V)^2$$

which yields a value of  $E(B - V) \approx 0.38 \pm 0.08$ . A higher value of  $E(B - V) = 0.6$  has been reported<sup>6</sup> from measurements made of both the interstellar K and  $D_2$  lines. Recently, a lower limit of  $E(B - V) = 0.18$  was determined from linear polarisation measurements<sup>2</sup>; a value of  $E(B - V) = 0.3 (+0.2/-0.1)$  was also reported<sup>1</sup> from an observation of the diffuse interstellar feature at  $\lambda 6614$ .

The data given in Table 1 are tabulated for 16 stars<sup>7</sup> in the field near the nova and are used to derive the distance–reddening relationship shown in Fig. 3. The solid line is representative of the general trend. The exact form of the relationship becomes increasingly uncertain beyond 1 kpc due to the paucity of stars with large colour excesses.

A values of  $E(B - V) = 0.38$  indicates that the nova is located at a distance of  $r \approx 3.3 \pm 0.6$  kpc where the error is estimated from the uncertainty in the distance–reddening law. The visual absorption corresponding to this colour excess is found to be



**Fig. 2** The K I ( $\lambda 7699 \text{ Å}$ ) interstellar feature ( $W_\lambda \approx 116 \text{ mÅ}$ ) as observed on 16.36 September 1978 (UT).  $\bullet$ , Observed points; Fourier fit indicated by solid line.

**Table 1** Reddening in the field of Nova Cygni 1978

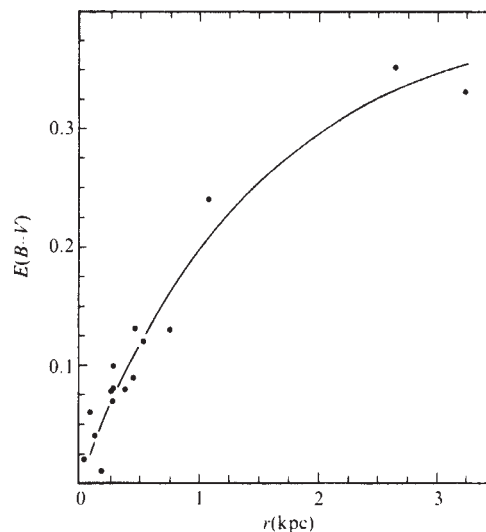
| Star*       | V    | $M_V$ | Spectral type† | $E(B - V)$ | $d(\text{pc})$ |
|-------------|------|-------|----------------|------------|----------------|
| HD204995    | 8.66 | -1.1  | B9(V)          | +0.13      | 751            |
| HD205601    | 6.76 | -1.4  | B6 V           | +0.08      | 377            |
| HD206280    | 6.72 | +0.5  | B9 V           | +0.01      | 172            |
| HD208513    | 7.65 | +0.2  | A0(V)          | +0.07      | 277            |
| HD208835    | 7.50 | -0.1  | A0(V)          | +0.10      | 291            |
| HD208878    | 7.43 | -0.1  | B8(V)          | +0.08      | 292            |
| HD209469    | 7.22 | -0.2  | B9(V)          | +0.08      | 269            |
| HD204153    | 5.60 | +3.0  | F0(V)          | +0.02      | 33             |
| HD204536    | 6.85 | -1.8  | B3 III         | +0.13      | 461            |
| HD205496    | 7.99 | -0.6  | B9(V)          | +0.09      | 454            |
| HD208962    | 7.60 | -1.4  | B9(V)          | +0.12      | 544            |
| HD209932    | 6.44 | +0.9  | B9 V           | +0.04      | 121            |
| HD209993    | 6.16 | +1.2  | A3 V           | +0.06      | 90             |
| HD204710    | 6.95 | -5.6  | B8 Ib          | +0.33      | 3,236          |
| HD204722    | 7.66 | -2.5  | B2 V           | +0.24      | 1,076          |
| BD +47°3588 | 9.61 | -2.5  | B1.5 V         | +0.35      | 2,642          |

\* Ref. 7.

† Luminosity class in parentheses assumed.

$A_v \approx R \cdot E(B - V) \approx 1.1 \text{ mag}$  (assuming  $R = 3.1$ ) and indicates a value of the corrected apparent visual magnitude at maximum light to be  $V_0 = m_v - A_v \approx 5.1 \text{ mag}$ . Combined with the above estimate for the distance, this implies a value of  $M_{V_0} \approx -7.5 \pm 0.5 \text{ mag}$  was reached by the nova at maximum light.

Since the time of maximum, the nova has declined at a rate of  $\sim 0.13\text{--}0.17 \text{ mag d}^{-1}$ . Using the speed classes defined by



**Fig. 3** The distance–reddening law in the direction of the nova. The solid points represent the data from Table 1; the line indicates the form of the reddening curve which is only approximately represented beyond 1 kpc.

McLaughlin<sup>8</sup>, Nova Cygni 1978 appears as a fast galactic nova, comparable to other fast novae such as El Aql ( $M_V \sim -7.35$ ) and DI Lac ( $M_V \sim -7.3$ ). Assuming a value  $B \sim 20$  for the nova precursor<sup>9</sup> implies an absolute magnitude for the precursor of  $M_B \sim 7 \text{ mag}$ .

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## Is Crab nebula a disrupted helium star?

WE propose here that the Crab nebula was produced by the explosion of a helium star in a close binary already containing a neutron star. This explosion would be caused by a complete spiralling of the neutron star into the helium star, which was totally disrupted<sup>1</sup>. The neutron star would be spun up in the process and be identified with PSR0532.

Arnett has pointed out that the overabundance of helium with respect to hydrogen in the Crab filaments<sup>2</sup> is compatible with a presupernova helium core of mass  $4 M_{\odot}$  (ref. 3) which corresponds to a main sequence mass of  $12 M_{\odot}$  (ref. 4). Chevalier<sup>5</sup>, on examining old Chinese records, present day nebula and the embedded pulsar, finds that all available information is consistent with the Crab having a massive progenitor. The fact that the motion of the Crab pulsar is directed away from the I Gem association has led to the suggestion<sup>6</sup> that the progenitor of the Crab was a runaway star from I Gem. According to this model, the first supernova explosion in a close binary in I Gem disrupted the binary releasing a neutron star (identified with PSR0527) and a normal star of mass  $12 M_{\odot}$  which later exploded to give the Crab nebula and the pulsar.

The model of a massive progenitor for the Crab as discussed above presents some problems. First, detailed work on the evolution of close binaries in the context of compact X-ray sources has shown (see ref. 1) that the first supernova explosion in a mass transfer binary is unlikely to disrupt the system. (If the binary is sufficiently wide so that mass transfer does not take place, the velocity of the normal star at the time of the disruption of the binary—equal to its orbital velocity—would be very small). Thus we are faced with the problem of placing a massive progenitor for Crab at  $|z| = 200$  pc. Second, to avoid an observable overabundance of heavy elements in the Crab filaments, it must be assumed<sup>3</sup> that the explosion of the helium star leaves behind a  $1.45 M_{\odot}$  neutron star core. Now this value is substantially larger than  $0.5 M_{\odot}$  estimated for the Crab pulsar on the basis of detailed models of neutron stars and data on micro- and macroglitches<sup>7</sup>. Third, there is the crucial problem of the mass. For the nebula a total mass of  $\sim 1 M_{\odot}$  has been estimated from the absolute intensities of emission lines<sup>8</sup>. On the other hand, Davidson *et al.*<sup>9</sup> conclude that a nebular mass in the range  $0.3$ – $10 M_{\odot}$  would not be inconsistent with observations, most of the mass being in dense neutral cores of filaments. However, the fact that bright and faint filaments have been accelerated equally, argues against the presence of too much neutral matter<sup>10</sup>. The mass of the nebula is therefore unlikely to be very much greater than  $1 M_{\odot}$ . Thus the problem is that if the Crab is a result of the supernova explosion of a massive star, where is the remaining mass?

These problems are solved if we assume that the progenitor of the Crab was a mass transfer binary. It is then obvious that the event of 1054 could not have been the first supernova explosion, because such an explosion would leave the binary intact. In other words the progenitor binary of the Crab must have passed through a Cen X3 type X-ray stage. The evolution of a normal massive star in a close binary containing a neutron star is characterised by a tremendous loss of mass and angular momentum<sup>11–13</sup>. The normal star expands and engulfs the neutron star, which leads to a rapid shrinkage of the orbit<sup>14</sup>. The spiralling in of the neutron star terminates when only the helium

core of the companion is left, the hydrogen rich envelope having been blown off<sup>13</sup>. For example<sup>15</sup>, starting with an X-ray binary consisting of a  $1 M_{\odot}$  neutron star and a  $15 M_{\odot}$  normal star, we may arrive at a system consisting of a  $1 M_{\odot}$  neutron star and a  $3 M_{\odot}$  helium star and having a period of  $\sim 1.5$  h corresponding to a separation of about  $1 R_{\odot}$ .

It has implicitly been assumed<sup>16,17</sup> that the helium star now evolves as a single star, so that its explosion in most cases disrupts the binary releasing two neutron stars, the old spun up neutron star manifesting itself as a type L pulsar and the newborn as a type D pulsar. However, conditions may be such that the helium star does not evolve as an isolated star. A  $3 M_{\odot}$  helium star has a radius of  $1.4 R_{\odot}$  at the onset of carbon ignition and of  $4.5 R_{\odot}$  at the onset of neon burning<sup>18</sup>. Thus if the separation between the two stars is sufficiently small, at some point in the evolution of the helium star the neutron star would be engulfed, triggering off a new spiralling in, which may totally disrupt the helium star and spin up the neutron star to very short periods<sup>1</sup>.

According to our hypothesis, the Crab nebula is such a disrupted helium star and the Crab pulsar, a spun up old neutron star (a type L pulsar). This model has many attractive features. It provides a helium star for explosion without an embarrassingly large mass for the nebula. It explains the observed overabundance of helium without postulating an excessively heavy neutron star or an unwanted excess of heavy elements. In this context it is relevant that Arnett<sup>3</sup> finds a better agreement between estimated<sup>3</sup> and inferred<sup>2</sup> abundances if interstellar matter is swept up rather than an old envelope (as would be the case if the exploding star were single).

The spun up neutron star in our model would move with a velocity equal to the centre of mass velocity the progenitor binary had acquired at the time of the formation of the neutron star<sup>1</sup>. (In refs 16, 17 it was assumed that a type L pulsar moves in the orbital plane of the progenitor binary). In the light of the above discussion, the fact that the motion of the Crab pulsar is directed away from the I Gem association permits a more plausible explanation (see ref. 6). The progenitor of the Crab was a massive close binary in I Gem. The first supernova explosion did not disrupt the binary, rather imparted the centre of mass a velocity of about  $125 \text{ km s}^{-1}$ . In about the 3 Myr between the first and the second explosion, the binary covered a distance of  $\sim 380$  pc—crossing the galactic plane—and appearing at the site of the explosion of 1054. (Note that in the model of Gott *et al.*<sup>6</sup>, the Crab pulsar did not acquire any velocity as a result of the supernova explosion).

The earlier suggestion<sup>17</sup> that PSR0532 is a type D pulsar and the associated type L pulsar should still be embedded in Crab nebula appears to be less attractive because of the problem of the abundance of heavy elements discussed above.

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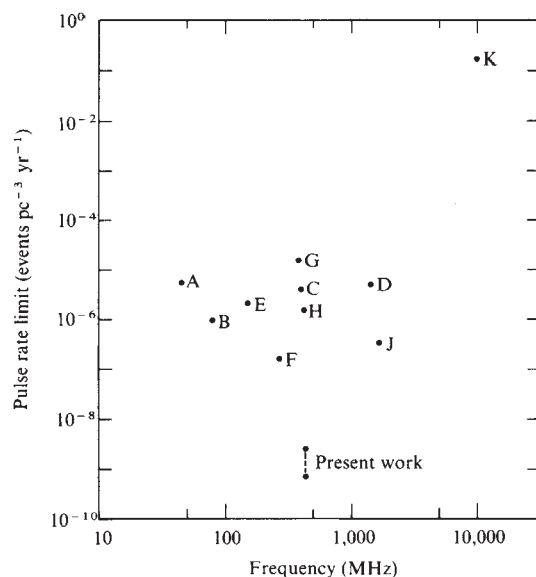
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## A sensitive search for radio pulses from primordial black holes and distant supernovae

THERE are two mechanisms of current theoretical interest which might generate short ( $< 1$  ms) radio pulses detectable at interstellar or even cosmological distances. In both cases the pulse is generated by the alteration of a magnetic field by an expanding conducting shell. The phenomenon might occur either as the expanding core of a supernova 'combs' the star's intrinsic dipole field<sup>1,2</sup>, or as the shell of charged particles emitted in the explosion of a primordial black hole (PBH) excludes the surrounding magnetic field<sup>3</sup>. In the latter case, the resulting radio pulse would, with suitable particle physics<sup>3,4</sup>, be much easier to detect than the  $\gamma$ -ray pulses that might also accompany the explosion<sup>5</sup>. The best published upper limit on the frequency of such  $\gamma$ -ray pulses is  $4 \times 10^{-2}$  events  $\text{pc}^{-3} \text{yr}^{-1}$  (ref. 6). We present here the results of a radio frequency search for isolated pulses, with much higher sensitivity than achieved previously. No definitive pulses have been detected, and the implied limit on PBH explosions is  $2 \times 10^{-9}$  events  $\text{pc}^{-3} \text{yr}^{-1}$ .

The results of several radio pulse searches have already been summarised, both from the point of view of supernova energies<sup>7</sup> and from that of limits on the number of PBH explosions<sup>8</sup>. Table 1 expands a summarising table from ref. 7, and gives the parameters of the most sensitive and most fully documented previous searches. We have attempted to compute the derived parameters as uniformly as possible, with a minimum of assumptions about the nature of the unseen pulses. Figure 1 shows the relative limits on the rate of pulse-producing events provided by the various surveys, plotted as a function of observing frequency.

Most of the published pulse searches have been carried out with simple receiving equipment and antenna apertures of not more than a few square metres. To achieve a greatly improved sensitivity limit, we have re-analysed data from a pulsar survey<sup>9</sup> done at the Arecibo Observatory in 1973–74. At 430 MHz the Arecibo telescope has an effective aperture of over 40,000  $\text{m}^2$ , and the pulsar survey made use of a special dispersion-compensating radiometer that permits a relatively broad total bandwidth to be used without losing sensitivity to impulsive signals. Relevant parameters of the Arecibo observations are summarised in Table 2.



**Fig. 1** Approximate limits on the rate of PBH explosions implied by the survey sensitivities listed in Table 1. The lower point plotted for the present work represents the limit as given in Table 1, computed as for the other surveys; the upper point is a firm,  $8\sigma$  limit based on the data given in Table 2.

Dispersion compensation was done by a small computer that sampled each of the 32 receiver output channels every 16.7 ms and then recombined them with progressive delays to 'de-disperse' the signals. In the observations under discussion the dispersion range was 0–640  $\text{cm}^{-3} \text{pc}$  (in steps of 20  $\text{cm}^{-3} \text{pc}$  and with time resolution of 16.7 ms) and 0–1,280  $\text{cm}^{-3} \text{pc}$  (steps of 40  $\text{cm}^{-3} \text{pc}$ , time resolution of 33.3 ms). The de-dispersed data were arranged in blocks of 8,192 samples (4,096 for the high-dispersion range), and for each block a two-dimensional histogram was printed showing the distribution of detected, de-dispersed signal amplitudes, as a function of dispersion measure. The observed frequency distributions were indistinguishable from a gaussian curve over the range of amplitudes for which adequate statistics were available ( $\pm 5$  standard deviations of the noise), and were also indistinguishable from those produced when a calibration noise source was inserted into the system.

About 12,000 blocks of data were searched for evidence of narrow pulses of well defined, non-zero dispersion measure.

**Table 1** Sensitivity parameters for 11 radio pulse surveys

| 1                 | 2                        | 3                              | 4                                 | 5                                  | 6                            | 7                            | 8                                                                       | 9                                              | 10                                                               |
|-------------------|--------------------------|--------------------------------|-----------------------------------|------------------------------------|------------------------------|------------------------------|-------------------------------------------------------------------------|------------------------------------------------|------------------------------------------------------------------|
| Survey            | Frequency<br>$\nu$ (MHz) | Bandwidth<br>$\Delta\nu$ (MHz) | Integration<br>time<br>$\tau$ (s) | System<br>temperature<br>$T_s$ (K) | Solid angle<br>$\Omega$ (sr) | Observing<br>time<br>$T$ (h) | Threshold<br>sensitivity<br>$U_{\min} (\text{J m}^{-2} \text{Hz}^{-1})$ | Maximum distance<br>for PBH<br>$d_{\max}$ (pc) | Rate of PBH<br>events<br>$R$ ( $\text{pc}^{-3} \text{yr}^{-1}$ ) |
| A <sup>11,7</sup> | 45                       | 1                              | 0.6                               | 25,000                             | 1                            | 1,768                        | $1.1 \times 10^{-21}$                                                   | 140                                            | $5 \times 10^{-6}$                                               |
| B <sup>12,7</sup> | 80                       | 1                              | 0.6                               | 5,000                              | 1                            | 1,100                        | $1.2 \times 10^{-22}$                                                   | 280                                            | $1 \times 10^{-6}$                                               |
| C <sup>13,7</sup> | 400                      | 0.06                           | $1.7 \times 10^{-5}$              | 200                                | 3                            | 100                          | $1.4 \times 10^{-22}$                                                   | 270                                            | $4 \times 10^{-6}$                                               |
| D <sup>13,7</sup> | 1,400                    | 0.5                            | $4 \times 10^{-6}$                | 150                                | 0.25                         | 100                          | $1.4 \times 10^{-23}$                                                   | 580                                            | $5 \times 10^{-6}$                                               |
| E <sup>14,7</sup> | 151                      | $\sim 1$                       | $\sim 1$                          | 1,000                              | $\sim 0.8$                   | 3,620                        | $3.4 \times 10^{-23}$                                                   | 160                                            | $2 \times 10^{-6}$                                               |
| F <sup>16</sup>   | 270                      | $50 \times 0.25$               | 0.02                              | 200                                | 1                            | 213                          | $1.1 \times 10^{-24}$                                                   | 900                                            | $1.6 \times 10^{-7}$                                             |
| G <sup>17</sup>   | 380                      | 3                              | 0.5                               | 500                                | 0.086                        | 232                          | $4.7 \times 10^{-24}$                                                   | 430                                            | $1.6 \times 10^{-5}$                                             |
| H <sup>18</sup>   | 430                      | 1.5                            | 0.1                               | 1,100                              | $3.3 \times 10^{-4}$         | 33                           | $3.2 \times 10^{-26}$                                                   | 5300                                           | $1.6 \times 10^{-6}$                                             |
| J <sup>19</sup>   | 1665                     | 15                             | 0.002                             | 200                                | $5.6 \times 10^{-5}$         | 28                           | $6.6 \times 10^{-28}$                                                   | $3.7 \times 10^4$                              | $3 \times 10^{-7}$                                               |
| K <sup>20</sup>   | 10,000                   | 40                             | 0.5                               | 20,000                             | $1.5 \times 10^{-3}$         | 1,700                        | $6.2 \times 10^{-22}$                                                   | 38                                             | $1.9 \times 10^{-1}$                                             |
| Present work      | 430                      | $2 \times 32 \times 0.25$      | 0.017                             | 175                                | $6.6 \times 10^{-6}$         | 292                          | $1.3 \times 10^{-29}$                                                   | $2.6 \times 10^5$                              | $7 \times 10^{-10}$                                              |

Data given in columns 2–7 were taken directly (or estimated) from the original references or from Meikle and Colgate<sup>7</sup>. The  $6\sigma$  threshold sensitivities (column 8) were computed from the relationship  $U_{\min} = 6(2kT_s)\Delta t A^{-1}(\tau\Delta\nu)^{-1/2}$  where  $k$  is Boltzmann's constant,  $A = \lambda^2/\Omega$  is the effective antenna aperture,  $\Delta t$  is the apparent pulse width expected at the receiver output, and the remaining quantities are defined in the table headings. For surveys A–D,  $\Delta t$  will be dominated by dispersion broadening for interesting dispersion measures; the quoted value of  $U_{\min}$  assumes  $\Delta t = 8,280 DM \Delta\nu^{-3}$  for  $\nu, \Delta\nu$  in MHz and  $DM = 100 \text{ cm}^{-3} \text{pc}$ . For the remaining surveys  $U_{\min}$  is independent of dispersion measure because  $\Delta t$  is dominated by  $\tau$ . We have not allowed for differences in antenna efficiencies or the threshold  $\sigma$  values, because few authors gave these. The entries may thus be in error by a factor  $\sim 2$ . Column 9 gives the maximum distance at which a PBH event of  $E = 10^{16} \text{ J Hz}^{-1}$  could be detected; for surveys A–D we assume a mean density of  $0.03 \text{ cm}^{-3}$  for the electrons causing dispersion along the line of sight. Finally, column 10 gives the corresponding upper limit on the rate of PBH events. For surveys A–D,  $d_{\max} \propto E^{1/3}$  and  $R \propto E^{-1}$ , while for the others  $d_{\max} \propto E^{1/2}$  and  $R \propto E^{-3/2}$ .



Blocks with broad, high intensity features at low dispersion measures were rejected as containing terrestrial interference. On all other blocks, all 'events' at or above  $6\sigma$  were catalogued. The frequency of pulses at the  $6\sigma$  level was consistent with that expected from random noise fluctuations.

No pulses were found at, or above, the  $8\sigma$  level, but eight unusual features seen at about 7 times the r.m.s. noise should be mentioned. These events do not seem to be mere statistical fluctuations, and most were found at such high apparent dispersion measures and so isolated that a plausible source of interference is not apparent. However, as they were evenly distributed in dispersion measure, and none appeared at or above 8 times the r.m.s. noise, it seems unlikely that many of them could have been produced by isolated events spaced uniformly over the region surveyed. They are not due to normal pulsars, because the original data was examined for periodicity ( $0.033 < P < 3.9$  s) by a computer program which could detect pulsars well below 6 times the r.m.s. noise, and some of them are clearly due to single events that lasted less than the integration time. The pulses might be due to irregular giant bursts from very faint pulsars, or to some completely unknown terrestrial or extraterrestrial cause. A decision on the source would require either vast amounts of observing time with a single Arecibo-sized antenna, or a shorter period with two Arecibo-sized telescopes operating simultaneously.

The sensitivities and pulse rates quoted in Table 1 are based on a  $6\sigma$  detection threshold and on the assumption that the pulses being sought are shorter than the integration times of the surveys. This assumption seems valid as the pulses produced by the mechanism mentioned earlier are expected to be intrinsically shorter than  $10^{-6}$  s, and broadening of the pulses by interstellar scintillation, if it is like that observed for pulsars<sup>10</sup>, should not be a problem, at least out to  $DM \approx 500 \text{ cm}^{-3} \text{ pc}$  (the largest dispersion yet observed for pulsars).

To make a rough estimate of the upper limit on the rate of PBH explosions implied by these observations, we assume that every explosion releases nearly  $mc^2$  of energy over a bandwidth of 1 GHz. With the assumptions introduced by Rees<sup>3</sup> and Blandford<sup>4</sup>, and taking the mass of still-unexploded black holes to be  $m_{\text{crit}} \approx 10^8 \text{ kg}$ , we find that the explosion releases energy density  $E \approx 10^{16} \text{ J Hz}^{-1}$  at frequencies near  $\nu_c \approx 1 \text{ GHz}$ . If the detection threshold is  $U_{\text{min}}$ , such an outburst could be detected to a distance  $d_{\text{max}}$  given by

$$U_{\text{min}} = E / (4\pi d_{\text{max}}^2). \quad (1)$$

The volume  $V$  surveyed is then

$$V = 1/3 d_{\text{max}}^3 \Omega \quad (2)$$

where  $\Omega$  is, say, the solid angle of the area of sky surveyed to better than  $-3\text{dB}$  of the on-centre sensitivity. The limit on the rate is then taken to be  $R = (VT)^{-1}$ , these rates are plotted in Fig. 1. The reader is invited to multiply them by the factor appropriate to his favourite distribution and confidence level. Corrections for  $E \neq 10^{16} \text{ J Hz}^{-1}$  may also be applied.

One must be careful in comparing the rates thus obtained, as there are many hidden assumptions (besides the one that the particle physics is such that the phenomenon can occur at all): first, the spectrum of the hypothetical pulses has been assumed flat from over the frequency range covered by these surveys; this is not likely to be the case<sup>4</sup>, but because the value of  $\nu_c$  is uncertain by several orders of magnitude, it is not possible to make any useful corrections.

Second, the magnetic field strength at the sites of the explosions is assumed to be a few microgauss. This is likely to be an overestimate for survey J and the present work, where much of the volume searched was intergalactic. Again, useful corrections are not possible, because  $\nu_c$  and the strength of the intergalactic field are unknown.

Finally, the PBH's are assumed to be uniformly distributed throughout space. If they are assumed to be correlated with galaxies (into which they might have fallen), or anticorrelated

**Table 2** Parameters of the Arecibo search

|                                      |                                                      |
|--------------------------------------|------------------------------------------------------|
| Frequency                            | 430 MHz                                              |
| Bandwidth                            | $32 \times 0.25 \text{ MHz}$                         |
| Polarisations                        | 2 circular, summed                                   |
| Integration time                     | 0.0167 s                                             |
| Effective aperture                   | $4 \times 10^4 \text{ m}^2$                          |
| System noise temperature             | 175° K                                               |
| Regions searched                     | Aquila, Taurus                                       |
| Interference-free search time*       | 292 h*                                               |
| Threshold sensitivity* ( $8\sigma$ ) | $3 \times 10^{-29} \text{ J m}^{-2} \text{ Hz}^{-1}$ |

\* Values quoted are for the lower dispersion-measure range. The higher  $DM$  data blocks had threshold sensitivity  $4 \times 10^{-29} \text{ J m}^{-2} \text{ Hz}^{-1}$  and were interference-free for 368 h.

(they might rapidly accrete to masses  $> m_{\text{crit}}$  inside galaxies) comparisons become much more difficult.

Any further interpretation of the data in Tables 1 and 2 must await theoretical developments.

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## A wind driven shell model for the absorption line systems in QSOs

THE spectra of many QSOs are rich in narrow absorption lines. The still somewhat uncertain identifications suggest that the absorption is due to ions of ionisation potential  $< 40 \text{ eV}$ . If the ionisation is due to the radiation from a central source, then theoretical models<sup>1–4</sup> give gas temperatures of  $< \sim 30,000 \text{ K}$ . The absorption line systems are generally blueshifted with respect to the emission line redshift of the object. If these blueshifts are Doppler shifts then they correspond to velocities ranging from  $\sim 0.001$  to  $0.5$  of  $c$  (the velocity of light)<sup>5</sup>. The interpretation of these differential redshifts has been controversial<sup>5</sup>. Opinion has polarised about two extreme hypotheses—that all the absorption is due to intervening galaxies (the intervening hypothesis) or that it all arises in the vicinity of the QSO (the intrinsic hypothesis). At present, apparently the only way of mapping the Universe at large distances is by observing QSOs. The weakness of current models for intrinsic absorption has led many to suppose that all the absorption occurs in intervening galaxies. If true, this has profound implications for the structure of the Universe. In particular, it implies that in the early Universe, galaxies had extremely large haloes (perhaps as large as  $0.5 \text{ Mpc}$  (ref. 6)). Furthermore, it suggests that there also existed, at high  $z$ , large clusters of galaxies with velocity dispersions much greater than those hitherto observed at small  $z$ .

(refs 5, 7, 8). Because of the importance of these conclusions for galactic structure and evolution, it is obviously essential to determine what proportion, in fact, of the absorption systems are intrinsic to the QSOs. We present here an intrinsic model which seems to explain the essential features of many absorption systems. This is a crucial first step towards the eventual separation of the observations into intrinsic and intervening systems.

The parameters given directly by the observations are the column density, ionisation state, velocity and velocity dispersion. It is also possible to estimate indirectly the density of the absorbing material<sup>9</sup> and its distance from the central radiation source<sup>9-11</sup>. Although uncertain<sup>12</sup>, these estimates suggest that the absorption takes place in thin, dense ( $\sim 10^{-3} \text{ cm}^{-3}$ ) shells at distances of the order of kpc (ref. 5).

All previous attempts<sup>13-15</sup> to explain these systems as intrinsic have relied on expulsion of gas from a central object. The principal difficulty with such models is that they are unable to produce enough absorbing material at large enough distances from the source. They also cannot account for the apparently normal abundances observed.

It is now generally believed that QSOs are active nuclei of galaxies and it is therefore reasonable to suppose that the absorbing material consists of interstellar gas which has been swept up by winds emanating from QSOs. Such a model gains added credibility from detailed investigations of the physics of radiatively accelerated winds<sup>15,16</sup> which show that it is inevitable that a significant fraction of the radiative energy output of a QSO is converted into kinetic energy of an outflowing wind<sup>16</sup>.

If a QSO emits a wind with velocity  $V$ , and with a mass-loss rate  $\dot{m}$ , into a uniform stationary gas of density  $\rho_0$ , then the flow will be as shown in Fig. 1. This flow pattern is set up because  $V$  is much greater than the sound speed in the ambient gas. The validity of the hydrodynamic approximation is discussed elsewhere<sup>17</sup>. This situation is entirely analogous to the interaction of a high speed stellar wind with interstellar gas<sup>18,19</sup>. In the initial stages of the flow both the shocked ambient and wind material

are far too hot either to cool or to give rise to absorption lines. In fact it can be demonstrated that the shocked QSO wind material behaves adiabatically over all time-scales of interest<sup>17</sup>. The effects of radiation pressure on the dynamics of the system can also be shown to be negligible during this phase<sup>17</sup>. During this completely adiabatic phase the position,  $r_s$ , of the outer shock can be described by the following similarity law:

$$r_s = \alpha \left[ \frac{1}{2} \frac{\dot{m} V^2}{\rho_0} \right]^{1/5} t^{3/5} \quad (1)$$

where  $\alpha$  is a dimensionless constant which is found from computations<sup>19</sup> to be equal to 0.88. A similarity solution is valid because the characteristic length,  $l = (\dot{m}/(V\rho_0))^{1/2}$ , is small compared with the typical scale size of the flow for all reasonable values of the parameters. For the swept-up material to be visible in absorption, it must cool. Cooling sets in suddenly<sup>19</sup> when the radiative cooling rate exceeds the expansion cooling rate immediately behind the outer shock ( $S_2$ ). This occurs at a time  $t_c$  when the shock has a radius  $r_c$  and velocity  $V_c$  given by<sup>17</sup>:

$$t_c = 4.3 \times 10^6 \dot{E}_{46}^{3/11} n_0^{-8/11} \text{ yr} \quad (2)$$

$$r_c = 7.7 \dot{E}_{46}^{4/11} n_0^{-7/11} \text{ kpc} \quad (3)$$

$$V_c = 3.6 \times 10^{-3} \dot{E}_{46}^{1/11} n_0^{1/11} c \quad (4)$$

In these equations the mechanical energy output rate,  $\dot{E} = \frac{1}{2} \dot{m} V^2$  of the QSO is expressed in units of  $10^{46} \text{ erg s}^{-1}$ . In deriving these equations, the cooling rate has been taken to be  $L = 1.68 \times 10^4 n T^{-1/2} \text{ erg g}^{-1} \text{ s}^{-1}$ . This is a reasonable approximation<sup>20</sup> for the temperature range,  $5 \times 10^4 \text{ K} < T < 5 \times 10^7 \text{ K}$ , and is calculated on the assumption of normal solar abundances. This is the appropriate temperature range for the onset of cooling in all circumstances which have been considered<sup>17</sup>. (The above parameters are not, in fact, very sensitive to the numerical coefficient adopted in the cooling rate. If  $L$  is multiplied by a factor  $A$ , say, it can be shown that  $t_c \sim A^{-5/11}$ ,  $V_c \sim A^{2/11}$ , and  $r_c \sim A^{-3/11}$ .)

Rather than attempting to present here a complete description<sup>17</sup> of results applicable to a wide variety of particular objects, we will now concentrate on the absorption system in the QSO 3C191. We do so because this object was not only the first QSO with identified absorption but is to date one of the very few QSOs which possesses an unambiguously identified set of absorption lines<sup>5</sup>. Studies of the spectra<sup>11</sup> place the absorption line system with a relative velocity of  $0.0027 c$  at a distance of  $\sim 10 \text{ kpc}$  from the source, and estimate a column density of  $N_{\text{HI}} \sim 3 \times 10^{19} \text{ cm}^{-2}$  and a number density  $\sim 10^3 \text{ cm}^{-3}$ . The observed visual luminosity,  $L_v$ , is  $10^{47} \text{ erg s}^{-1}$  (assuming  $H_0 = 50$  and  $q_0 = 0$ ).

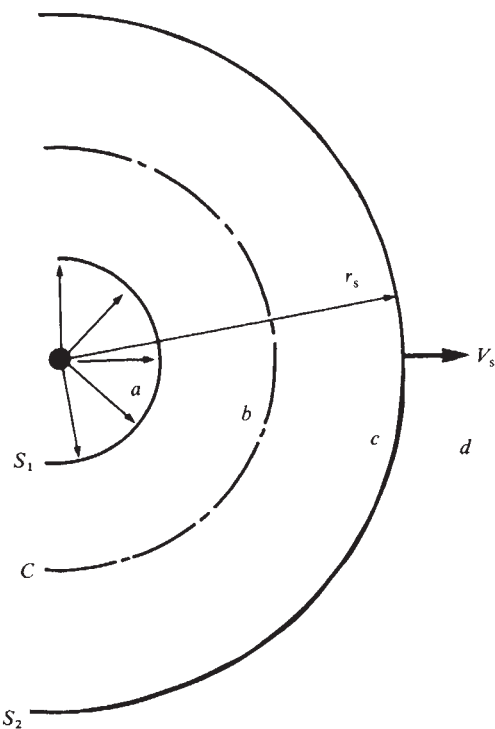
We will suppose that the spectrum is flat ( $\alpha \sim 1$ ) with a drop or cut off at about the He II ionisation limit. Theoretical studies<sup>16</sup> show that a value of  $\dot{E} \sim 10^{45} \text{ erg s}^{-1}$  is consistent with this spectrum and the observed visual luminosity. For  $n_0$  we adopt the value of 1, but note that the results are rather insensitive to the actual choice of parameters. These values give  $t_c = 2.3 \times 10^6 \text{ yr}$ ,  $r_c = 3.3 \text{ kpc}$ ,  $V_c = 0.0029 c$ . At this shock speed, the postshock temperature is  $\sim 10^7 \text{ K}$ . The gas will now cool according to the cooling law above until it reaches a state of ionisation and thermal equilibrium with the radiation field. The equation of state is now<sup>15,21</sup>

$$P = R\rho y f(y) \quad (5)$$

where

$$y = L_v/(4\pi r^2 \rho c^3) \quad (6)$$

is the ionisation parameter<sup>14,21</sup>,  $R$  is the gas constant, and  $f(y) \equiv T(y)/(\mu y)$ . This state is critically dependent on the spectrum of the QSO<sup>21</sup>. For flat spectra  $F(\nu) \sim \nu^{-\alpha}$  (with  $\alpha \sim 1$ ) the equilibrium temperature is a few times  $10^4 \text{ K}$ . Calculations<sup>21</sup> of the photoionisation equilibrium show that over many orders of magnitude in  $y$  for most spectra, the gas is quasi-isothermal at



**Fig. 1** Flow pattern of a QSO: *a*, the freely outflowing wind; *b*, the shocked wind; *c*, swept up ambient gas; *d*, undisturbed ambient gas.  $S_1$  is an inwards facing shock in the wind,  $C$  is a contact discontinuity and  $S_2$  is the outwards moving shock in the ambient gas.



either  $\sim 10^6$  K (high ionisation) or  $\sim 10^4$  K (medium ionisation to neutral). In these quasi-isothermal states a good approximation<sup>15,21</sup> for  $f(y)$  is  $f(y) \sim By^{-\beta}$ , with  $\beta \sim 1$ ,  $B(10^6 \text{ K}) \sim 6 \times 10^5$ ,  $B(10^4 \text{ K}) \sim 6 \times 10^3$ . As the gas cools  $V$  will initially decrease, and an upper limit to the postshock pressure is

$$P_s = \frac{2}{\gamma + 1} \rho_0 V_c^2 \quad (7)$$

Then the number density in the cool material is

$$n \leq n_0 \frac{V_c^2}{R y_c f(y_c)} \quad (8)$$

where  $y_c$  is the equilibrium value of the ionisation parameter. Substituting equations (6) and (2) into equation (8), we obtain the implicit equation for  $y_c$ :

$$f(y_c) \leq 9.3 \times 10^{15} n_0^{-1/11} E_{46}^{10/11} L_{v,46}^{-1} \quad (9)$$

$$(L_{v,46} \equiv L_v/10^{46})$$

For the values of  $n_0$ ,  $E$  and  $L_v$  for 3C191, we find that  $\log_{10} f(y_c) \approx 14.1$ . From Rösner's<sup>21</sup> calculations we find  $\log y_c \approx -9.5$ . For this spectrum, at  $y_c$ , C IV and Si IV are the dominant ions. During the subsequent evolution<sup>17</sup>  $y$  decreases so that the gas becomes more neutral. From  $y_c$  and  $f(y_c)$  we find that (equation (8))  $n \approx 2.3 \times 10^3$ .

The shell thickness,  $\Delta r$ , is given by

$$\Delta r = \frac{1}{3} \frac{n_0}{n} r_s \quad (10)$$

so that  $\Delta r/r \approx 10^{-4} \ll 1$ . (The importance of this small ratio is that it has always been supposed difficult to get such a small ratio in an intrinsic model.) The column density in neutral hydrogen,  $N_{\text{HI}} \sim X(1-x)N_{\text{H}}$ , where  $N_{\text{H}} = n_0 r_c$  and  $x = x(y)$  is the degree of ionisation of hydrogen, and  $X$  is the mass fraction of hydrogen. At  $y_c$ ,  $(1-x(y_c)) \sim 10^{-4}$ , so that  $N_{\text{HI}} = 10^{18} \text{ cm}^{-2}$ . Since  $y$  decreases,  $N_{\text{HI}}$  will increase.

All these values are in surprisingly good agreement with the observations in view of the crudity of this first model. This agreement can be shown to remain even when we consider non-uniform and non-spherically symmetric ambient density distributions<sup>17</sup>.

Once a cool dense shell has formed, it will start to absorb momentum from the radiation field.

We do not discuss the further behaviour of the shell here in detail, because, in contrast with the formation of the shell, the flow depends strongly on the parameters. We show<sup>17</sup> that in the majority of cases, the shell accelerates due to the radiative driving and that often the acceleration is limited only by the total momentum content of the radiation field. If this happens, then it is unavoidable that the shell becomes subject to the radial instabilities discussed elsewhere<sup>22</sup>, as well as non-radial instabilities<sup>17</sup>. The more effective the radiation driving, the higher the velocity and the more unstable is the system. Thus the mechanism seems capable of providing a natural explanation for the multiplicity of high velocity systems. In addition, if the shell does accelerate, then there will be some gas which cannot cool, and will be collisionally ionised, which might explain the apparently anomalous appearance of ions of high ionisation potential in otherwise relatively neutral systems.

We have also examined such important questions as the effects of varying the spectral distribution of the radiation field and of departures from spherical symmetry. These considerations place definite constraints on the characteristics of the radiation sources and on the gas distribution in galaxies in order that absorption line systems form by the mechanism discussed above. Note that the removal of gas by the wind will inevitably have important consequences for the further evolution of the galaxies.

Whereas we do not believe that this mechanism can explain all the absorption line systems of QSOs, our detailed exploration of its consequences<sup>17</sup> should provide a basis for distinguishing between intrinsic and intervening systems.

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## Measurement of forces between two mica surfaces in aqueous poly(ethylene oxide) solutions

WE have measured the forces between two curved mica surfaces immersed in a solution of 0.01% poly(ethylene oxide) (molecular weight 148,000) in 0.04 M  $\text{MgSO}_4$  (a good solvent). The experimental technique allows the measurement of (1) forces as a function of surface separation; and (2) the refractive index of the medium between the surfaces<sup>1,2</sup>. The forces exhibited large irreversibilities as well as short-term and long-term time dependent effects, and we conclude that—for observation times less than a few hours—these interactions cannot be described in terms of an equilibrium force law without specifying the previous history of approach of the surfaces.

Figure 1 shows results obtained after 2 h equilibration. The arrows indicate the paths taken in measuring the forces ( $A_1 \rightarrow B_1 \rightarrow C_1 \rightarrow A_2 \rightarrow \dots$ ). Each compression-decompression cycle ( $A_n \rightarrow B_n \rightarrow C_n$ ) took 5–15 min, during which the surfaces were approached to point  $B_n$  and then separated to a large distance,  $>300$  nm, and after a time brought together again ( $A_{n+1} \rightarrow B_{n+1} \rightarrow \dots$ ). Three distinct force regimes were distinguished, which we label  $\alpha$ ,  $\beta$  and  $\gamma$ .

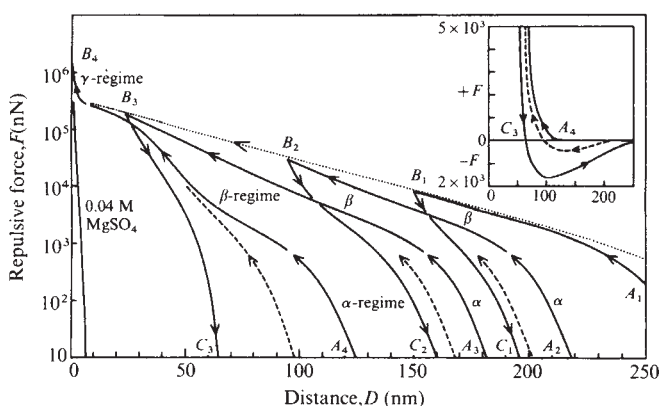
$\alpha$ -Regime: if the surfaces are kept well separated after a decompression for more than 10 min the initial repulsion on approach rises sharply and roughly exponentially, and its onset depends on the closest distance of previous approach (Fig. 1). However, if the surfaces are kept well separated for a few hours

the forces always tend to the limiting force law (Fig. 1, dotted line). The repulsive forces in the  $\alpha$ -regime extend over 15–30 nm, are reversible over short time periods (a few minutes), and have the same form irrespective of the onset separation. This regime may possibly be associated with the 'diffuse interpenetration' domain discussed by Napper<sup>3</sup>. (We also note that if the surfaces are approached within only 1 or 2 min after a decompression from a small separation the repulsion may be preceded by an attractive region. See Fig. 1, inset.)

**$\beta$ -Regime:** on further approach the repulsion increases less rapidly and the force varies roughly linearly with distance, but closer in it rises more rapidly. The  $\beta$ -regime is highly unstable and irreversible: the force depends on the rate of approach, and if the surfaces are kept under a constant force they drift towards each other at 1–5 nm min<sup>-1</sup>, though closer in the inward drift rate is lower. We have also observed that the surfaces can undergo sudden jumps towards each other, suggestive of the 'yield' characteristic of polymeric gels possessing a network structure undergoing partial collapse<sup>4</sup>. If the externally applied force is decreased in the  $\beta$ -regime the surfaces move out only very gradually or stick, indicating adhesion. The behaviour of the forces in the  $\beta$ -regime may be due to (1) slow adsorption (desorption) processes, including bridging, of tails during compression (decompression); (2) a slow reconfiguration of a polymeric network undergoing rearrangement of intertail crosslinks. The attractive forces measured during and soon after a decompression from a small separation also strongly suggests that significant bridging occurs.

**$\gamma$ -Regime:** below ~5 nm, the repulsion increases very steeply and polymer now becomes forced out from between the surfaces, which ultimately come into molecular contact within  $\pm 0.05$  nm of the original contact measured in MgSO<sub>4</sub> solution without polymer.

We have also measured the refractive index of the aqueous polymer medium between the surfaces at various separations. The refractive index  $n$  rises as the separation falls towards the  $\gamma$ -regime, thereby indicating that in the  $\alpha$ - and  $\beta$ -regimes polymer is being compressed, though some extrusion may also be occurring. Typical values are:  $n = 1.336 \pm 0.003$  at 45 nm,  $n = 1.343 \pm 0.003$  at 11 nm. As for bulk polymer solution:  $n =$



**Fig. 1** Repulsive forces  $+F$  measured between two crossed cylindrical surfaces of mica of radius  $\sim 1$  cm as a function of separation  $D$  in 0.01% poly(ethylene oxide) in 0.04 M MgSO<sub>4</sub> at 21 °C. The force measured before addition of poly(ethylene oxide) is shown on the extreme left and corresponds to pure double-layer repulsion and van der Waals attraction<sup>2,8</sup>. The limiting force with poly(ethylene oxide) (dotted line) is attained a few hours after addition of polymer (for an approach rate of  $\sim 10$  nm min<sup>-1</sup>). After compression ( $A_n \rightarrow B_n$ ) and decompression ( $B_n \rightarrow C_n$ ), the subsequent compression forces measured  $\sim 1$  min later are shown as dashed curves, while those measured 10–20 min later are shifted farther out ( $A_{n+1} \rightarrow B_{n+1}$ ). The inset shows detail on a linear plot of the decompression ( $B_3 \rightarrow C_3$ ) and subsequent compressions ( $A_4 \rightarrow B_4$ ) measured 1 and 15 min, respectively, after decompression  $C_3$ .

1.334, and for pure poly(ethylene oxide):  $n = 1.454$ , these results indicate that the number of adsorbed polymer chains per surface is  $\sim 2 \times 10^{11}$  cm<sup>-2</sup>—a similar value to that obtained for the equilibrium adsorption of high molecular weight poly(ethylene oxide) on silica<sup>5</sup>.

The magnitude of the forces was different when different micas were used, probably reflecting the different adsorption capacity of poly(ethylene oxide) on different mica surfaces. However, the qualitative features discussed here were always the same, and forces were always measured at distances many times  $\langle r^2 \rangle^{1/2}$  ( $\sim 30$  nm) of the poly(ethylene oxide), qualitatively similar to recent results obtained with poly(vinyl acetate)<sup>6,7</sup>.

Our results indicate (1) the existence of a dilute long-range polymer layer forming on mica surfaces, probably possessing gel-like properties; (2) that even though the forces may be monotonically repulsive on approach, once significant overlap occurs there is sticking, probably through polymer bridging, resulting in effective adhesion; and (3) as the rate of segmental rearrangement towards equilibrium is very slow ( $> 1$  h) the effective forces between colloidal particles with adsorbed polymers of high molecular weight may often be determined by non-equilibrium relaxation mechanisms.

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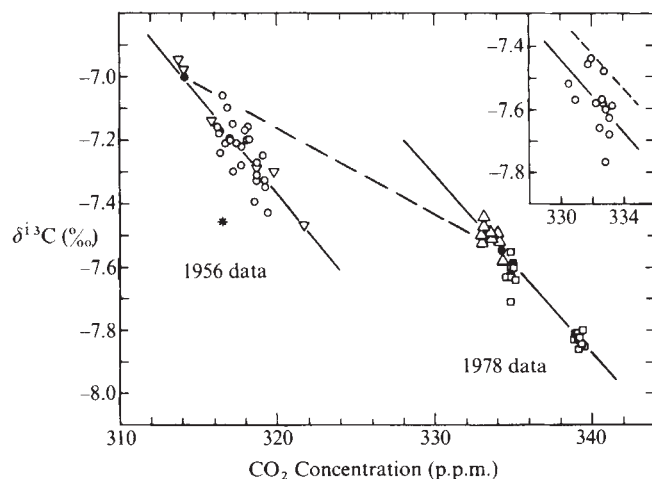
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## Recent trends in the <sup>13</sup>C/<sup>12</sup>C ratio of atmospheric carbon dioxide

THE <sup>13</sup>C/<sup>12</sup>C ratio of atmospheric carbon dioxide has decreased by approximately 0.6‰ over 22 yr according to new direct measurements reported here. Our results offer a way of establishing whether <sup>13</sup>C/<sup>12</sup>C ratios of tree rings<sup>1–6</sup> are representative of atmospheric <sup>13</sup>CO<sub>2</sub> variations. We have carried out both isotopic (at Groningen) and concentration (at La Jolla) measurements of atmospheric CO<sub>2</sub> on air samples obtained during 1977 and 1978 at three widely spaced locations: La Jolla, California (33°N, 117°W), Fanning Island (4°N, 159°W) and the South Pole. Sampling, instrumental, and analytical procedures closely matched a similar study carried out 22 yr earlier by Keeling<sup>7,8</sup>.

The new isotopic measurements are plotted in Fig. 1 as the per mil variation,  $\delta^{13}\text{C}$ , of the <sup>13</sup>C/<sup>12</sup>C ratio from the historic standard PDB. Also plotted are isotopic data for 1955 and 1956 for those sites and times of day of the original study having minimal local disturbance to the CO<sub>2</sub> concentration from vegetation. Published 1955–56  $\delta^{13}\text{C}$  values have been recomputed from the original mass spectrometer mass ratios, 45/44 and 46/44, to be consistent with the computational methods used for the 1977–78 data. Corrections for valve mixing and <sup>17</sup>O





**Fig. 1** Change in the relation between carbon isotopic ratio  $\delta^{13}\text{C}$ , and concentration of atmospheric  $\text{CO}_2$  over 22 yr. Mean change is shown as a dashed line. Locations as follows:  $\circ$ , Inyo Mountains; \*, Inyo sample omitted in computing equation (2);  $\nabla$ , three west coast United States sites shown for comparison (only samples collected nearest noontime are plotted), Olympic Beach<sup>7</sup>, Yosemite National Park<sup>8</sup>, Organ Pipe National Monument<sup>8</sup>;  $\triangle$ , Fanning Island;  $\square$ , La Jolla, California. Solid lines show mixing relations for 1956 and 1978 as given by equations (2) and (1), respectively.  $\bullet$ , denote inferred Northern Hemisphere means for 1956 and 1978. Inset: relationship of  $\delta^{13}\text{C}$  to concentration for the South Pole. Solid line shows mixing relation assuming same slope as Northern Hemisphere (shown dashed.)

follow prescribed methods<sup>9</sup>. The new measurements are calibrated against NBS-20 and a set of organic standards in use since 1963 (ref. 10), whereas the 1955–56 measurements are against PDB itself and two other carbonate standards previously calibrated against PDB. Recomputation has lowered the 1955–56  $\delta^{13}\text{C}$  values by 0.09 to 0.10‰. Details of this recomputation, and instrumental and analytical details of the new measurements, will be published elsewhere.

The calibration accuracy of the 1955–56 data is estimated to be within  $\pm 0.13\%$ ; that of the new data  $\pm 0.05\%$ .

As seen in Fig. 1, the 1977–78 Northern Hemisphere samples irrespective of latitude closely follow a single mixing equation<sup>7</sup> (in per mil):

$$\delta = -26.54 + 6346.98/\mu \quad (1)$$

where the first term denotes the  $\delta^{13}\text{C}$  of  $\text{CO}_2$  added or subtracted from the sampled air by either fossil fuel combustion or plant activity (which cannot be distinguished);  $\mu$  is the  $\text{CO}_2$  concentration in p.p.m. of dry air. We adopt a provisional estimate of the seasonally adjusted concentration for Mauna Loa Observatory (unpublished) as representative of the Northern Hemisphere for 1 January 1978; thus  $\mu_{78} = 334.2$  p.p.m. It follows from equation (1) that  $\delta_{78} = -7.55\%$ . A similar analysis for the Southern Hemisphere (South Pole data) yields  $\delta_{78} = -7.59\%$  for  $\mu_{78} = 332.6$  p.p.m. Thus no appreciable mean isotopic gradient appears to exist between hemispheres in spite of a small gradient in the seasonally adjusted concentration.

For 1955–56 the Inyo Mountain series ( $37^\circ\text{N}$ ,  $118^\circ\text{W}$ ) of March 1956, are the most extensive set of data for a single location. These data obey the mixing equation:

$$\delta = -26.97 + 6271.63/\mu \quad (2)$$

To obtain a representative value of  $\mu$  we extrapolated backwards from early 1958 when the first precise concentration measurements were obtained at Mauna Loa Observatory<sup>11</sup> on the assumption that the  $\text{CO}_2$  varied in proportion to the known input from fossil fuel between 1956 and 1978. For January 1, 1956 we find  $\mu_{56} = 314.1$  p.p.m. It follows from equation (2) that  $\delta_{56} = -7.01\%$ .

The above isotopic ratios for both 1956 and 1978 are influenced by atmospheric  $\text{N}_2\text{O}$  which is included in the sample gas analysed by the mass spectrometer<sup>12</sup>. To correct for this we assumed a present  $\text{N}_2\text{O}$  concentration of 0.30 p.p.m. and a 1.5% increase in  $\text{N}_2\text{O}$  per decade<sup>13</sup>. The correction is +0.32% in 1956 and +0.31% in 1978.

We find that the composition of atmospheric  $\text{CO}_2$  over the Northern Hemisphere has changed from 1956 to 1978 as follows:

$\text{CO}_2$  concentration ( $\mu$ ): 314.1 to 334.2 p.p.m.

carbon-13 ratio ( $\delta$ ): -6.69 to -7.24‰

For the Southern Hemisphere no isotopic data exist for 1956. Concentration data since 1958 (ref. 14) and the present isotopic data indicate, however, that differences between both hemispheres cannot be large compared to hemispheric changes in 22 yr. We therefore regard the Northern Hemisphere data as being close to global averages.

The observed decrease in  $\delta^{13}\text{C}$  over 22 yr agrees to the stated accuracy with predictions of the effect of fossil fuel combustion on the carbon cycle<sup>15</sup> based on two recently published geochemical models<sup>16,17</sup>. In summary, these models yield almost identical predictions if the land biosphere is assumed to respond to the recent atmospheric  $\text{CO}_2$  rise<sup>17</sup> such that the observed increase (20.1 p.p.m. or 43 Gton C) is satisfied. When the isotopic fractionation factors (kinetic air to sea, 0.986; equilibrium air to sea, 1.009; air to biosphere, 0.982; air to fossil fuel, 0.980) are treated without mathematical approximation<sup>18</sup>, the decrease in  $\delta^{13}\text{C}$  is 0.44‰ for a fossil fuel  $\text{CO}_2$  input of 79 Gtons (ref. 17 and unpublished data). This result assumes that the biosphere grew in 22 yr by 12 Gtons. The model predictions, however, are insensitive to biospheric growth which can even be assumed negative. For example, the models predict a change of 0.42‰ if the biosphere shrunk by 40 Gtons, attended by a much greater oceanic uptake of  $\text{CO}_2$  than assumed in the first calculation (76 instead of 24 Gtons).

This circumstance suggests that a time record of atmospheric  $^{13}\text{CO}_2$  data cannot help decide the rate of biospheric growth or shrinkage during this 22 yr period of concentration measurements. Such a record may, nevertheless, be a valuable substitute before the time of reliable direct concentration measurements. Our results are offered as an aid in verifying tree ring studies which span both the past and recent periods.

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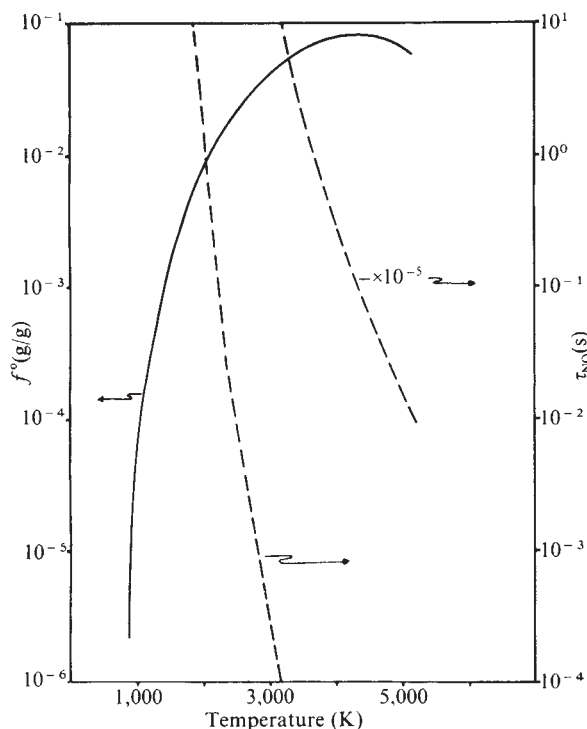
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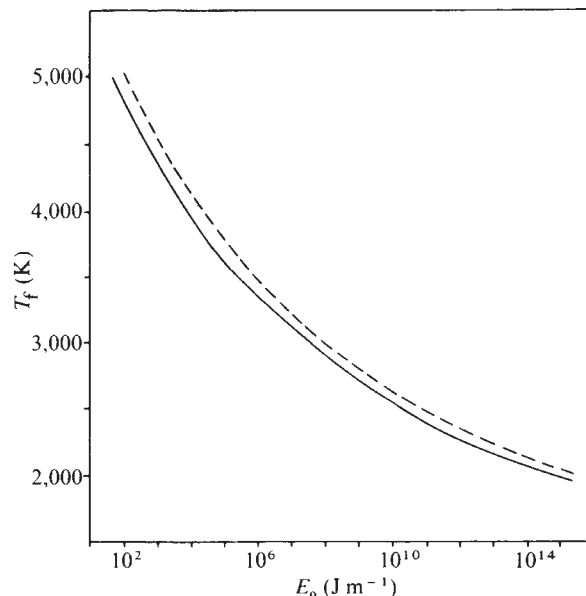
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## Effect of variable energy input on nitrogen fixation in instantaneous linear discharges

ATMOSPHERIC nitrogen oxides are a central issue in the photochemistry of the troposphere, stratosphere and urban atmosphere. The removal of nitrogen oxides by precipitation is a source of fixed nitrogen to the biosphere. Thus the nitrogen fixation rate by natural and anthropogenic discharges is of wide concern<sup>1-8</sup>. While it is generally assumed that  $P$ , the molecules of NO produced per joule of discharge, is independent of the input energy, the analysis reported here implies that  $P$  does vary with input energy. Thus care must be taken when extrapolating NO yields from small laboratory sparks to more energetic processes of potential geochemical significance. Although the calculations



**Fig. 1** The NO equilibrium mass mixing ratio,  $f^o$ , represented by the solid line, and the NO chemical lifetime,  $\tau_{NO}$ , represented by the dashed line, as a function of temperature,  $T$ . Thermochemical data from the JANAF tables<sup>9</sup> was used to determine  $f^o$  from an initial mixture of 0.78 atm  $N_2$ , 0.21 atm  $O_2$ , 0.01 atm  $H_2O$ ,  $3.3 \times 10^{-4}$  atm  $CO_2$ , and  $1.5 \times 10^{-6}$  atm  $CH_4$ , and ambient temperature of 288 K, and an assumed  $n_M$ , number density within the heated air, of  $2.5 \times 10^{19}$  molecules  $cm^{-3}$ . The equilibrium concentrations as a function of  $T$  were determined for  $N$ ,  $N_2$ ,  $NH_x$  ( $x = 1, 2, 3$ ), NO,  $NO_2$ , O,  $O_2$ ,  $O_3$ , H,  $H_2O$ ,  $H_2$ , HCN, OH,  $HO_2$ , CO,  $CO_2$ ,  $CH_x$  ( $x = 1, 2, 3, 4$ ),  $N_2O$  and HNO. The rates of the reactions  $NO + N \rightarrow N_2 + O$ ,  $NO + O \rightarrow N + O_2$ , and  $NO + NO \rightarrow N_2O + O$  were used to determine  $\tau_{NO}$  with kinetic data obtained from Baulch *et al.*<sup>10</sup> and Hampson and Garvin<sup>11</sup>.



**Fig. 2** The freeze-out temperature,  $T_F$ , as a function of discharge energy,  $E_0$ , as determined from equation (8). The solid line is for  $\gamma = 1.4$  and the dashed line is for  $\gamma = 1.25$ .

assume a cylindrical symmetry appropriate for a linear discharge, the results are consistent with equivalent calculations using spherical symmetry.

As identified by Zel'dovich<sup>1,2</sup>,  $N_2$  and  $O_2$  are converted to NO by high temperature chemical reactions occurring in the shock-heated air surrounding the discharge. Figure 1 shows  $f^o$ , the equilibrium NO mass mixing ratio, and  $\tau_{NO}$ , the NO chemical lifetime, as a function of temperature,  $T$ , for an initial mixture of air at 1 atm and 288 K. Defining  $n_M$  as the number density of the heated air,  $\tau_{NO}$  is accurately represented by

$$\tau_{NO} = \frac{1.55 \times 10^{-2} \exp(53,942/T)}{n_M^{1/2}} \quad (1)$$

(Calculations indicate that for the region of interest  $n_M$  is 1-10 times the ambient number density. As  $n_M$  increases,  $f^o$  slowly decreases.)

If a parcel of air is heated to  $T \geq 4,000$  K and cooled at a rate characterised by a cooling time,  $\tau_T(T)$ , NO will remain in equilibrium until  $T = T_F$ , the 'freeze-out' temperature, where

$$\frac{\tau_T(T)}{\tau_{NO}(T)} \begin{cases} > 1 \text{ for } T > T_F \\ = 1 \text{ for } T = T_F \\ < 1 \text{ for } T < T_F \end{cases} \quad (2)$$

For  $T < T_F$ , NO is too long-lived to adjust to the decreasing temperature and NO is frozen at  $f^o(T_F)$ . The NO yield is approximated by

$$P = \frac{M(T_F, E_0) f^o(T_F)}{E_0} \frac{6.02 \times 10^{23}}{30 \times 10^{-3}} \quad (\text{molecules } J^{-1}) \quad (3)$$

where  $M$  is the mass ( $kg m^{-1}$ ) of air heated above  $T_F$  and  $E_0$  is the input energy ( $J m^{-1}$ ).

To evaluate  $\tau_T$  and  $M$  we adopt the numerical-difference equations derived by Lin<sup>12</sup> which simulate a strong shock produced by an instantaneous linear discharge. While these equations do not directly consider translational, rotational, vibrational and chemical modes of energy transfer, by assuming specific heat ratios,  $\gamma$ , of 1.4 and 1.25, these effects should be adequately bounded<sup>13</sup>. For an ambient pressure,  $p_0$ , of 1,013



mbar and density,  $\rho_0$ , of  $1.228 \text{ kg m}^{-3}$ ,  $T^1$ , the shock front temperature, is

$$T^1 = A(\gamma) \frac{E_0^{1/2}}{t} \quad (\text{K}) \quad (4)$$

where  $t$  is the time in seconds after the discharge and

$$A(\gamma) = \begin{cases} 1.11 \times 10^{-4} & \text{for } \gamma = 1.4 \\ 6.18 \times 10^{-5} & \text{for } \gamma = 1.25 \end{cases} \quad (5)$$

Following Zel'dovich and Raizer<sup>1</sup>, we define  $\tau_T$  at  $T^1 = T_F$  as

$$\tau_T(T_F) = \left( \frac{1}{T^1} \frac{dT^1}{dt} \right)^{-1} \bigg|_{T^1=T_F} \quad (6)$$

from equation (4)

$$\tau_T(T_F) = \frac{AE_0^{1/2}}{T_F} \quad (7)$$

Thus  $\tau_T$  increases as  $E_0$  increases, or the cooling rate decreases as the surface-to-volume ratio decreases.

Combining equations (1), (2) and (7), for  $n_M = 2.5 \times 10^{19} \text{ cm}^{-3}$ ,

$$E_0 = \left[ \frac{3.0957 \times 10^{-12}}{A} T_F \exp(53942/T_F) \right]^2 \quad (\text{J m}^{-1}) \quad (8)$$

Figure 2 illustrates the resulting inverse relationship between  $T_F$  and  $E_0$  for  $\gamma = 1.4$  and  $1.25$ . While  $T_F \sim 2,000 \text{ K}$  for  $E_0 \sim 10^{14} - 10^{15} \text{ J m}^{-1}$ , for lower energies  $T_F > 2,000 \text{ K}$ . These results contradict previously held assumptions that  $T_F \sim 2,000 \text{ K}$ , independent of  $E_0$ , and, as outlined below, implies that  $P$  varies with  $E_0$ .

From Lin's<sup>12</sup> equations,

$$M(T_F, E_0) = \pi R^2(T_F, E_0) \rho_0 = \frac{C(\gamma) E_0}{T_F} \quad (\text{kg m}^{-1}) \quad (9)$$

where  $R$  is the shock front radius and

$$C(\gamma) = \begin{cases} 3.95 \times 10^{-4} & \text{for } \gamma = 1.4 \\ 1.71 \times 10^{-4} & \text{for } \gamma = 1.25 \end{cases} \quad (10)$$

Substituting in equation (3)

$$P = C(\gamma) \frac{f_0(T_F)}{T_F} \frac{6.02 \times 10^{23}}{30 \times 10^{-3}} \quad (\text{molecules J}^{-1}) \quad (11)$$

The dependence on  $E_0$  is caused by the variation of  $T_F$  with  $E_0$ . Because  $R^2 \propto 1/\rho_0$ ,  $P$  is not directly dependent on  $\rho_0$  or  $p_0$ ; the variation of  $\tau_T$  and  $f^\circ$  with ambient conditions results in only a weak dependence of  $P$  on  $p_0$  and  $\rho_0$ .

Figure 3 depicts  $P$  for  $\gamma = 1.4$  and  $1.25$  as well as some laboratory results<sup>7,14,15</sup>, which are consistent with the variations predicted here. The equivalent NO yields for megaton point explosions determined by previous investigators<sup>3-6</sup> which are included in Fig. 3 are also consistent with the present results.

As  $E_0$  decreases from  $10^{15}$  to  $10^4 \text{ J m}^{-1}$ ,  $f^\circ/T_F$  increases and thus  $P$  increases. For  $E_0 < 10^4 \text{ J m}^{-1}$ , however,  $T_F > 4,000 \text{ K}$ ,  $f^\circ$  maximises and then decreases, and thus  $P$  also decreases. Over the range  $10^1 - 10^{16} \text{ J m}^{-1}$ ,  $P$  varies by a factor of about 9 with a maximum near  $10^4 \text{ J m}^{-1}$ . Decreasing  $\gamma$  from  $1.4$  to  $1.25$ , reflecting an increase in heat capacity, causes a 50% decrease in  $P$ .

In addition to the above dimensional calculations, fully time-dependent calculations were carried out, similar to those of Goldsmith *et al.*<sup>5</sup>, to confirm the behaviour of  $P$ . Using Lin's<sup>12</sup> equations, the temperature and density histories of 11 mass shells (of  $10^{-5}$ ,  $10^{-3}$ ,  $2 \times 10^3$ , and  $2 \times 10^7 \text{ kg m}^{-1}$  for  $E_0 = 10^3$ ,  $10^5$ ,  $10^{11}$ , and  $10^{15} \text{ J m}^{-1}$ , respectively) extending radially outward were obtained. These parameters as a function of time were coupled to an  $\text{N}_2/\text{O}_2/\text{NO}$  chemical scheme<sup>5,10,11</sup> to determine the NO production within each shell. The results of these kinetic calculations are also indicated in Fig. 3. Because

the kinetic calculations included the enhanced densities of the shocked air while the dimensional calculations assumed  $n_M = 2.5 \times 10^{19} \text{ cm}^{-3}$ , the yields from the former method are somewhat lower than those from the dimensional approach. The finding from both calculations that  $P$  may vary by as much as an order of magnitude over the range of input energies is of potential geochemical significance. An illustrative example is presented below.

To estimate the fixation rate by atmospheric lightning, Chameides *et al.*<sup>7</sup> adopted a  $P$  of  $(6 \pm 1) \times 10^{16} \text{ molecules J}^{-1}$  on the basis of  $10^1 \text{ J m}^{-1}$  spark experiments and simple calculations assuming  $T_F \sim 2,000 \text{ K}$ . However, if a lightning stroke dissipates about  $10^5 \text{ J m}^{-1}$  (ref. 16), a value of  $(8-17) \times 10^{16} \text{ molecules J}^{-1}$  may be more appropriate, assuming that the highly idealised shock wave model used here adequately simulates the cooling of air around a lightning discharge. Taking  $1.6 \times 10^{-7} \text{ J cm}^{-2} \text{ s}^{-1}$  (ref. 7) as the total energy dissipated by lightning, we obtain a global NO production rate of  $(1-2.5) \times 10^{10} \text{ NO molecules cm}^{-2} \text{ s}^{-1}$  or  $(35-90) \times 10^{12} \text{ g N yr}^{-1}$ . This may be an overestimation if a large portion of the stroke energy is consumed by the leader or if multiple-stroke flashes do not additively increase

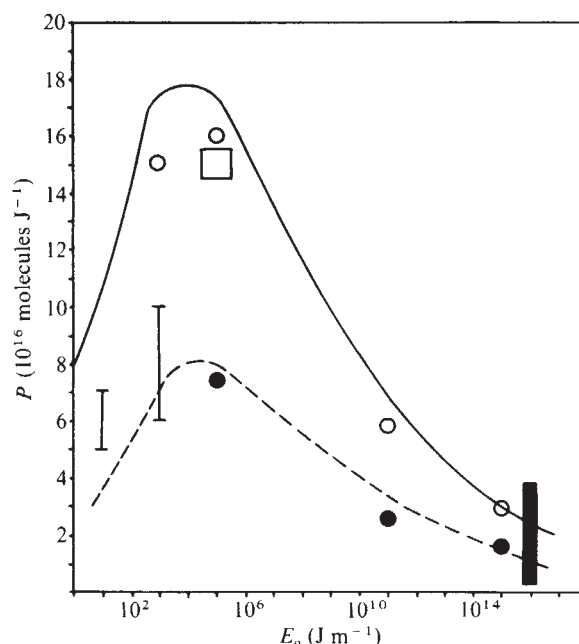


Fig. 3 The yield of NO,  $P$ , as a function of discharge energy,  $E_0$ . The solid line is for  $\gamma = 1.4$  and the dashed line is for  $\gamma = 1.25$ . The results of the fully time-dependent calculations for  $\gamma = 1.4$  (O) and  $\gamma = 1.25$  (●), are given. The vertical lines depict the experimental findings of Chameides *et al.*<sup>7</sup> and the square the results of the experiments of Zipf and Dubin<sup>14,15</sup>. The vertical rectangle indicates the equivalent results obtained by previous investigators using spherical geometry for explosions in the megaton range<sup>3-6</sup>.

the NO yield. For comparison, it is estimated that  $\sim 20-30 \times 10^{12} \text{ g N yr}^{-1}$  are produced by anthropogenic activities<sup>17,18</sup>. As nitrogen oxides are removed from the atmosphere at a rate of  $\sim 90 \times 10^{12} \text{ g N yr}^{-1}$ <sup>7,19,20</sup>, it is likely that lightning and combustion are the major sources of atmospheric nitrogen oxides<sup>18,21</sup>, both processes involving the conversion of  $\text{N}_2$  and  $\text{O}_2$  to NO by high temperature chemical reactions.

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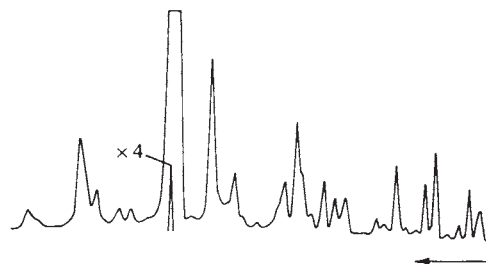


Fig. 1 Capillary gas chromatogram of the total sterols isolated from the Black Sea sapropel layer.

## Black Sea sterol—a molecular fossil for dinoflagellate blooms

A PECULIAR sterol has been found in recent sedimentary environments which we eventually found was identical with the Black Sea sterol. This sterol was first mentioned among the extractable sterols from the Black Sea sapropel layer as the dominant component<sup>1</sup>. The concentration of this sterol in these rare sediments seemed high enough for us to isolate and purify the object to determine its molecular structure and evaluate its significance. We have isolated and analysed the sterol fractions from the Black Sea sapropel (by techniques to be described elsewhere), but here only elucidate the molecular structure of the Black Sea sterol and discuss its significance.

The sediment investigated, which was deposited during the rise of the oxic-anoxic (H<sub>2</sub>S) interface after the last glaciation in the Black Sea (7,000–3,000 yr BP), has an organic matter content up to 50% and is composed of numerous membranous structures<sup>2</sup>. During this period the surface water of the Black Sea gradually changed from fresh water to marine conditions. This was accompanied by a changing phytoplankton population from fresh water diatoms and dinoflagellates to marine coccolithophores in the period after 3,000 yr BP (ref. 2). The oceanographic conditions in the brackish water period together with special pathways of organic matter degradation, transformation and preservation in the gradually more anoxic deep water, must have led to the deposition of the unusually organic rich sediment. The abundance of dinoflagellate cysts in the sediment points to an important role of these algae in the brackish water period, which is understandable considering the wide salinity tolerance of dinoflagellates<sup>3</sup>. We feel that the occurrence of the Black Sea sterol could be related to the postulated abundance of dinoflagellates in the surface waters during the period 7,000–3,000 yr BP. The knowledge of the exact structure of this peculiar and abundant sterol in the sediment must give clues to its biosynthetic origin and subsequently to the origin of the sedimentary organic matter. The sediment investigated was sampled by gravity corer in the Western abyssal basin of the Black Sea (41°39' N, 30°44' E, –2,150 m). The core (covered core length 118 cm) consisted of a white top layer (0–27 cm), a dark green microlaminated layer (27–71 cm) similar in appearance to the Unit 2 sediment of Ross and Degens<sup>4</sup>, and clayish sediments (71–118 cm). The section from 27 to 71 cm was received frozen for analysis. The sediment was homogenised in a Waring blender and divided into portions of

250 g. Total sterols were isolated from the extract of such a portion, obtained after saponification of the wet sediment by ethanolic KOH, followed by digitonin precipitation for isolation of the 3 $\beta$ -sterols. The digitonin precipitation procedure was carried out by addition of an excess amount of a 1% digitonin solution in 90% ethanol to the extract dissolved in C<sub>2</sub>H<sub>5</sub>OH–CH<sub>2</sub>Cl<sub>2</sub> (3:1, v/v).

After stirring for two hours at room temperature, the mixture was centrifuged and the precipitate washed thoroughly with ethanol. The precipitate was refluxed in pyridine for 30 min. Digitonin was precipitated from the reaction mixture by addition of diethyl ether. The diethyl ether–pyridine solution was treated with 2 M HCl and the ether layer, containing the freed 3 $\beta$ -sterols, was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The isolated sterol fraction was transformed into the corresponding trimethylsilyl ethers by BSA (bistrimethylsilylacacetamide, Pierce). Gas chromatographic separation was achieved on a Perkin Elmer 990 instrument, equipped with a 25 m glass capillary column (i.d. 0.25 mm) coated with OV 101. The temperature was programmed from 140 to 280 °C at a rate of 4 °C min<sup>–1</sup>. Helium was used as carrier gas. Figure 1 shows the appropriate part of the capillary gas–liquid chromatography trace of the sterol fraction. The major peak in this gas chromatogram, previously designated as 'Black Sea sterol', was the subject of a detailed structural analysis. Figure 2 shows the mass spectrum of the Black Sea sterol trimethylsilyl ether derivative, which was obtained by gas chromatography/mass spectrometry on a Hitachi MS 52 instrument equipped with a 42-m glass SCOT column (i.d. 0.75 mm) coated with OV 101.

The molecular ion *m/e* 500 points to a C<sub>30</sub> steroidal alcohol with one double bond equivalent. The fragment ions *m/e* 229, *m/e* 231, *m/e* 269 and *m/e* 271 indicate a ring saturated steroid skeleton with one 4-methyl group<sup>5</sup>. The mass spectrum is identical to the mass spectrum of the trimethylsilyl ether derivative of Dinosterol (4 $\alpha$ -methyl-5 $\alpha$ (H)- $\Delta^{22}$ -23,24-dimethyl-cholestan-3 $\beta$ -ol)<sup>6</sup>.

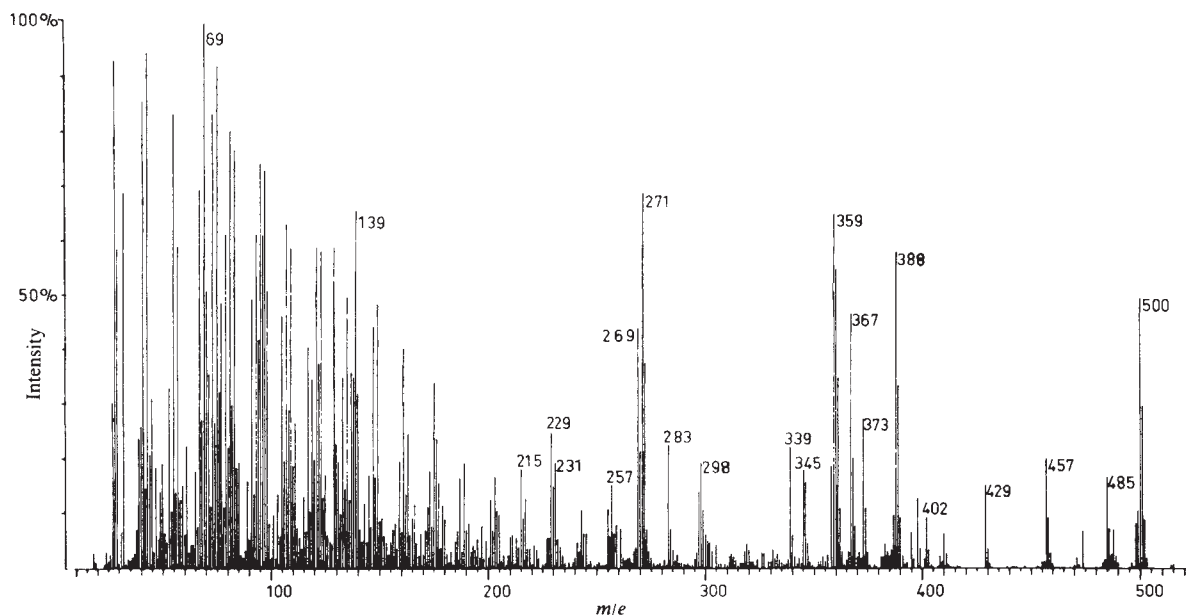
Further evidence for the possible similarity between the Black Sea sterol and dinosterol was obtained by comparative studies using <sup>1</sup>H NMR and high-resolution gas chromatography.

For that purpose the crude Black Sea sterol was purified further by preparative thin layer chromatography on silica gel with CHCl<sub>3</sub>–CH<sub>3</sub>OH (99:1, v/v) as eluent. Crystallisation of the purified sterol yielded 200  $\mu$ g of needles with a melting point of 220–220.5 °C. The melting point of dinosterol has been reported<sup>7</sup> to be 220–222 °C.

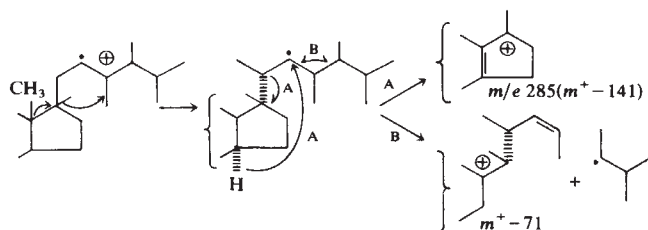
The <sup>1</sup>H NMR was measured on a Varian CFT-20 spectrometer in CDCl<sub>3</sub>. The spectrum of the Black Sea sterol was found to be identical with the one of dinosterol showing a vinyl proton at  $\delta$ 4.96 (1H, q, *J* = 1.1, 10 Hz) and vinylic methyl protons at  $\delta$ 1.59 (*J* = 1.1)<sup>6,7</sup>. High resolution gas chromatography was performed on a Perkin Elmer 990 equipped with glass capillary column on two different polymers (OV-101 and Carbowax high polymer). The retention times of the silylated derivatives of both sterols were identical on co-injection.

These data demonstrate that the Black Sea sterol possesses a structure identical to that of the dinosterol, being 4 $\alpha$ -methyl-5 $\alpha$ (H)- $\Delta^{22}$ -23,24-dimethyl-cholesten-3 $\beta$ -ol. As dinosterol has





**Fig. 2** Mass spectrum of the Black Sea sterol obtained by capillary GC-MS of the silylated sterol fraction. The  $\Delta^{22-23,24}$ -dimethyl unit in the side chain can be recognised in the mass spectrum by  $m^+ - 71$  and  $m^+ - 71 - 90$ . A fragmentation process is postulated in view of literature data<sup>15</sup>:



previously been encountered as the major sterol in some dinoflagellates only<sup>6</sup>, we assume that the abundance of this sterol in the extract of the sapropel layer of the Black Sea sediment reflects a relatively large contribution of dinoflagellate organic matter to this sediment. The remarkable richness in organic matter of the sapropel layer compared with the present marine deposits (Unit 1)<sup>4</sup> suggests periodic blooming of these algae in the brackish water ecosystem as the possible mechanism for organic matter supply, as otherwise similar conditions of

preservation and allochthonous sediment influx for both sediment types exist.

To establish the relative contribution of organic matter from different sources, an analysis of the bulk composition of the organic matter by Curie point mass spectrometry was undertaken<sup>8</sup>. This relatively new technique has demonstrated its merits in the analysis of bacteria, fungal cell walls, humic substances and kerogen<sup>9-12</sup>.

The pyrolysis mass spectrum of the sapropel (Fig. 3) shows the abundant occurrence of pyrolytic fragments typical of polysaccharides such as  $m/e$  32, 60, 72, 74, 82, 85, 96, 98, 102, 110, 112, 114, 126 and 128; and to some extent aminosugar polymers such as  $m/e$  95, 97, 109, 125, 137, 151.

Pyrolytic fragments typical of proteins such as  $m/e$  17, 34, 48, 67, 92, 94, 108, 117, 131, and lignin such as  $m/e$  94, 108, 124, 138, 150, 152, 164 are very low or even absent<sup>10,11</sup>.

The pyrolysis fragments  $m/e$  17, 34, 36, 38 and 48 are due to compounds such as ammonia, seasalt (HCl) and sulphur compounds ( $H_2S$  and  $CH_3SH$ ). The intense  $H_2S$  peak is usually encountered in sediments with active or fossil sulphur-reducing activity.

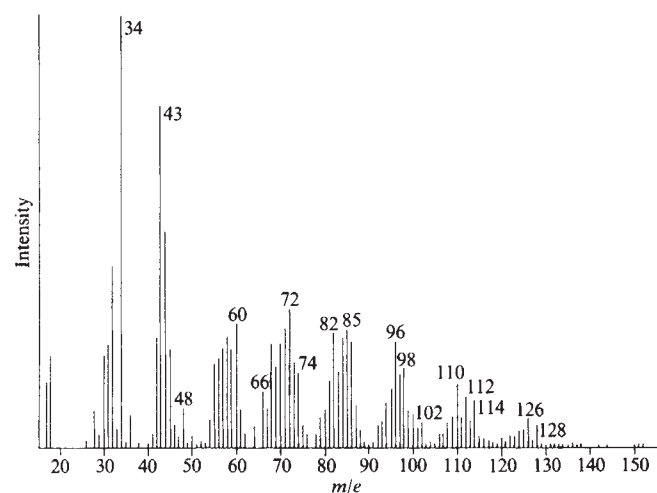
As the thecal plates of dinoflagellates are mainly composed of polysaccharides<sup>13</sup>, we think that the bulk of the Black Sea sapropel is mainly built up from dinoflagellate remains.

Contributions of bacterial organic matter previously based on the analysis of the sedimentary fatty acids<sup>14</sup>, is now substantiated by the presence of aminosugar polymers, which make up the cell walls of bacteria.

The Black Sea sapropel unit therefore should be considered as a dinoflagellate ooze, partly reworked by anaerobic bacteria.

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**Fig. 3** Curie-point pyrolysis mass spectrum of homogenised Black Sea sapropel. Pyrolysis temperature: 610 °C; ionisation voltage: 14 eV.

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## Elimination of natural scrapie in sheep by sire genotype selection

SCRAPIE, a subacute neuromuscular disease of middle-aged sheep, has been attributed variously to an infection or to heredity<sup>1,2</sup>. The clinical disorder follows progressive symmetrical decay of neurones in localised brain sites, notably the hypothalamo-neurophysial and olivo-pontocerebellar 'systems' (ref. 3), associated with a terminal axon dystrophy<sup>4–9</sup>. An artificially transmissible spongiform encephalopathic agent (TSEPA), or 'slow virus', to which many attribute the disease<sup>10,11</sup>, is commonly present in diseased animals but its aetiological role remains unclear. Based on the clinical manifestation of natural scrapie, I have proposed that the disease is controlled by an autosomal recessive gene<sup>1,2,12</sup>. This hypothesis allowed identification of the three presumptive genotypes (*ss*, *sS*, *SS*), and precise prediction of attack rates<sup>13,14</sup>, amenable to quantitative experimental verification<sup>15</sup>. The evidence summarised here, which has been compiled since 1958, provides strong support for my hypothesis.

The data have been collected from stud sheep flocks, each of 50–300 ewes, of 11 breeds in England and Scotland<sup>12,16</sup>; a 25-yr continuous record of one breeder group of 15–20 flocks with 2,500 ewes provides the principal data, which are supplemented by records of my own experimental flock of 250. Only 35–40% of the females and 1–2% of the males in the flocks have been kept past the age of 1½ yr. Half of these survive to 4½ yr and a few to 10 yr. Ten other breeder groups with 200 flocks have provided data over shorter periods, to give records on some 40,000 individuals.

The diagnosis of scrapie was based on (1) clinical signs<sup>2</sup>, (2) the family and clan health history<sup>2</sup>, (3) autopsy after euthanasia<sup>3</sup>, (4) 'blind' histopathological assessment by light microscopy<sup>3,12</sup> with occasional use of electron microscopy<sup>4–6</sup> and immunohistochemistry<sup>7–9</sup> and (5) occasional assay for the TSEPA. Of about 3,000 affected animals, I have observed 2,100 and autopsied 1,900, of which 1,040 were examined histopathologically, with only two disagreements with the other criteria.

As, in our population, 85–90% of all cases of scrapie were recognisable clinically by 4½ yr of age<sup>2</sup>, I took this as the minimum age for classification designated as follows. (1) 'Black': affected, or if unaffected, with both parents affected, that is, of presumptive homozygous recessive *ss* genotype; (2) 'grey': unaffected, but with one parent affected or with affected progeny, that is, of presumed heterozygous recessive *sS* genotype; (3) 'white': unaffected, with neither parent affected and with no affected progeny, that is, of probable homozygous dominant *SS* genotype, although a proportion, the progeny of grey × grey matings, will be heterozygotes; (4) 'proven whites': rams shown by test-mating to be free of the presumptive scrapie allele, that is, of 'proven' double-dominant genotype. The progeny generations sired without interruption by 'proven' rams are designated F<sub>1</sub>W, F<sub>2</sub>W and so on. Assuming the original F<sub>0</sub> dams to be of the three genotypes in equal numbers, then the chance that an animal of the F<sub>5</sub>W–F<sub>6</sub>W generations carries the scrapie allele will be about 1 in 100, and of the F<sub>8</sub>W–F<sub>9</sub>W generations about 1 in 1,000.

Rams were test-mated with 'black' and 'grey' ewes to provide sufficient progeny for at least 10 offspring out of 'black' ewes or 22 out of 'grey' ewes, or a suitable proportion of each dam category, to be observed for at least 4½ yr. Assuming that only 80% of potential cases of scrapie, the *ss*, manifest the disease by 4½ yr, as a more recent study indicates<sup>13</sup>, the probability that an allele-carrying ram in such a sire-progeny group will fail to produce an offspring affected within 4½ yr is <1%. A ram with no affected progeny is assumed not to carry the scrapie allele and is a 'proven white'. Of 105 rams tested in eight flocks, 59 had more than one affected progeny and were discarded; 46 had no affected progeny; of these, 18 have proved to be free of the allele at a level of *P* < 0.01 or less, 3 to *P* < 0.05–0.01, 4 are now under test, and for 17, the data are insufficient to provide an assess-

**Table 1** Scrapie among the progeny of affected and unaffected rams born in one flock at different periods over 23 yr

|                                                               | <i>a</i>      |            | <i>b</i>   |               |
|---------------------------------------------------------------|---------------|------------|------------|---------------|
| Birth cohorts                                                 | 1951–59       | 1966–73    | 1958–59    |               |
| Mean annual flock attack rate                                 | 11.0%         | 1.4%       | 14.9%      |               |
| Sires                                                         | 7 affected    | 8 affected | 4 affected | 1 allele-free |
| Total progeny retained/average life span (yr)                 | 242/4.7       | 103/5.2    | 85/4.2     | 21/7.5        |
| Total affected/affected plus unaffected at age 4½ yr or older | 109/195 (56)* | 2/71 (3)   | 45/71 (63) | 0/17          |
| Out of 'black' ( <i>ss</i> ) dams                             | 53/56 (95)    | 1/2 (50)   | 21/22 (96) | 0/10‡,        |
| Out of 'grey' ( <i>sS</i> ) dams                              | 42/86 (49)    | 1/25† (4)  | 18/35 (51) | 0/7‡,¶        |
| Out of 'grey'/'grey' dams                                     | 14/39 (36)    | —          | 6/12 (50)  | —             |
| Out of 'white' ( <i>SS</i> ) dams                             | 0/14§ (0)     | 0/44 (0)   | 0/2 (0)    | —             |

\* % Affected with scrapie.

† Under-manifestation.

‡ Two in each group left affected progeny in the F<sub>2</sub> generation.

§ Nine brains examined histologically and found free of lesions at age 9½–10 yr.

|| Nine brains examined histologically and found free of lesions at age 7–10½ yr.

¶ Six brains examined histologically and found free of lesions at age 7–10½ yr.

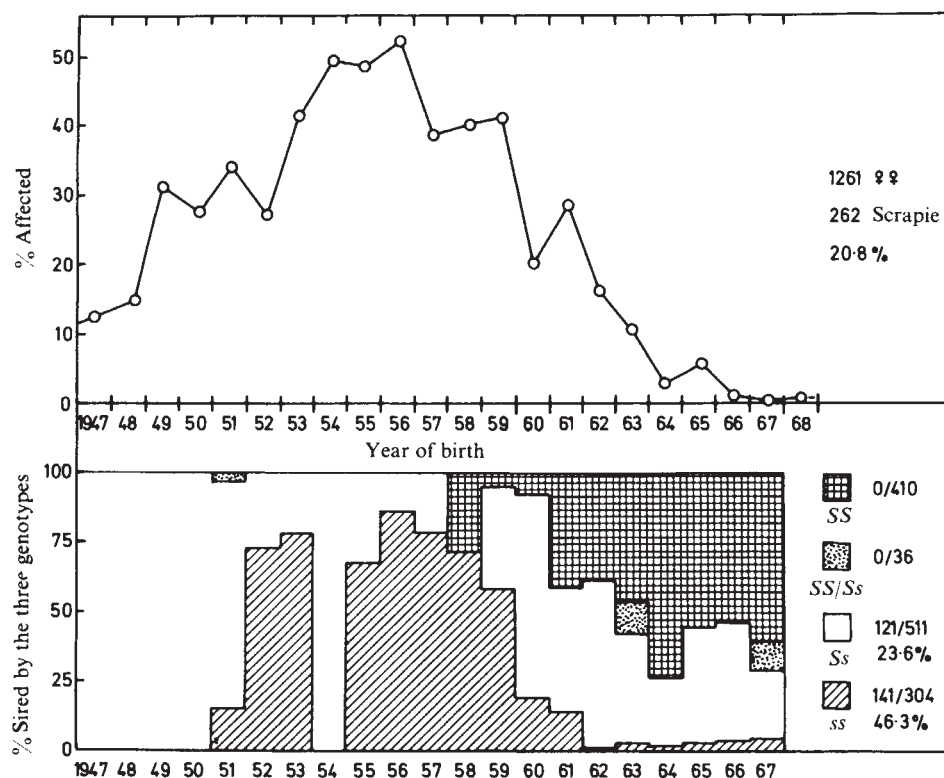


Fig. 1 The scrapie attack rate in the females of one flock for the period 1947-67 shown by annual birth cohorts in the curve (above) and according to the scrapie genotypes of their sires in the histogram (below).

ment, whereas in 4 cases test-mating was abandoned for other unrelated reasons. The 18 'proven white' rams were born and reared in 10 flocks, and were at stud in 3 flocks, which had scrapie present throughout the rams' lives. Ewes from 15 flocks, some with scrapie and some not, have been sent to these rams for mating.

This breeding programme confirmed three predictions of the genetic hypothesis<sup>2,13-15</sup>. (1) That no progeny of a 'proven white' ram should develop scrapie under any natural circumstances. 1,223 Progeny, 1,167 female and 56 male, of the 18 'proven white' rams were born and reared to adults in 15 flocks. 825 Females and 12 males were observed to 4½ yr or older, many to 7 yr, the oldest being 13½ yr. No case of scrapie has occurred in these sheep, although all 15 flocks contained affected animals during some part of the observation period, 1958-78. Sixty-three progeny were out of dams affected during their gestation or suckling, or manifesting scrapie within 2 yr; 46 were observed

to 4½ yr or older, with 25 observed to 7½ yr and some to 11 yr; no clinical signs of scrapie were observed. Eight of the oldest animals, which had lived continuously in contact with clinical scrapie, showed no microscopical evidence of scrapie or of the common nonspecific senile changes. Data on the progeny of the first 'proven white' ram, kept in a high incidence environment, are shown in Table 1b. (2) That cases will reappear in the F<sub>2</sub> backcrosses of 'grey' parents sired by 'proven white' rams. In the high incidence flock cited in Table 1, matings of two heterozygous F<sub>1</sub>W rams with known allele-carriers provided 21 progeny observed to 4½ yr or older, of which five developed scrapie. (3) That consecutive top crosses of 'proven rams' should so reduce the scrapie-allele frequency that the disease will disappear. 308 Females and 18 males of the F<sub>2</sub>W-F<sub>6</sub>W generations remain under observation and free of clinical scrapie. Figure 1 shows the gradual disappearance of scrapie from one severely affected flock following the retention of the progeny of 'proven white' rams. No case has occurred in the main flock since the 1968 cohort, although two females retained for ram test-mating and six introduced stud rams were affected up to 1976. One F<sub>5</sub>W ram carried the allele, derived from an affected great-great-great-grandmother and passed 'silently' without clinical illness through the intervening generations. In the breeder group, which has been using some 'proven white' rams and their sons since 1962, continuous data for 1953-78 are available in 13-18 flocks with 1,900-2,900 flock ewes. The attack rate was 0.4% in 1953-54, reached a peak of 3.2% (85 cases) in 1963-64, and then declined gradually to 0.5% in 1972-73, and 0.2% in 1976-77, with no new cases in home-bred sheep since 1977.

Figure 1 also shows that the recessive gene concept provided precise explanations of the variable occurrence of the disorder, given the genotypes of the dams. Table 1 amplifies this analysis during periods of high and low flock attack rates. The agreement with the mendelian predictions is very close. This is confirmed by the summary of the nine reciprocal matings shown in Table 2, those in section a being from the flock in Fig. 1, and those in section b from three other flocks of the same breeder-group and free of scrapie since 1953.

After a 20-yr breeding programme based on my genetic hypothesis, scrapie has disappeared in the breeder group as predicted. Although our affected sheep carried the scrapie

Table 2 Occurrence of scrapie by age 4½ yr among the progeny of the nine reciprocal matings of the three presumptive scrapie genotypes

| No. of sires | Parents' genotypes ♂♂ × ♀♀ | No. of progeny                          |                                |           |
|--------------|----------------------------|-----------------------------------------|--------------------------------|-----------|
|              |                            | Total affected plus unaffected to 4½ yr | Affected with scrapie Observed | Expected† |
| a            | ss × ss                    | 56                                      | 48                             | 48        |
|              | ss × Ss                    | 124                                     | 57                             | 53        |
|              | Ss × ss                    | 46                                      | 23                             | 20        |
| 7            | Ss × Ss                    | 106                                     | 18                             | 22        |
|              | SS × ss                    | 39                                      | 0                              | 0         |
|              | SS × Ss                    | 7                                       | 0                              | 0         |
|              | SS × SS*                   | 159                                     | 0                              | 0         |
| b            | ss × SS*                   | 156                                     | 0                              | 0         |
|              | Ss × SS*                   | 23                                      | 0                              | 0         |

\* Presumed very low s allele frequency based on family, flock and breeder group records over 20 yr.

† Based on assumption of 85% of total manifestation completed by age 4½ yr (ref. 2).



TSEPA, I doubt that it is ever communicable outside the laboratory. There is no good evidence for dissemination of the disease by a communicable agent<sup>10,11</sup>, by placental cannibalism<sup>17</sup>, by vertical transmission from the dam<sup>18</sup>, or through the action of a dominant susceptibility gene<sup>19,20</sup>. I conclude that in the sheep population studied and probably in most farm sheep in Britain (1) natural scrapie is determined, primarily, by an autosomal recessive gene expressed fully in both sexes; (2) no F<sub>1</sub> progeny of a 'proven' allele-free ram will develop scrapie, whatever the scrapie status of its dam or its exposure to cases of the disease; (3) the scrapie attack rates among the nine progeny groups derived from the reciprocal crosses of the three parental genotypes will conform to the predictions of Mendel's law; (4) the practical control of the disease, and its elimination if desired, may be achieved by the use of allele-free rams and the retention of their progeny; (5) the dissemination of natural scrapie does not depend on a naturally communicable parasitic agent; and (6) the hypothesis most consistent with present evidence<sup>2,10,15,16</sup> is that the scrapie TSEPA is formed *de novo* in each affected animal by the metabolic activity of the natural recessive gene<sup>2,16</sup>. A more detailed account of this work will be published elsewhere<sup>21</sup>.

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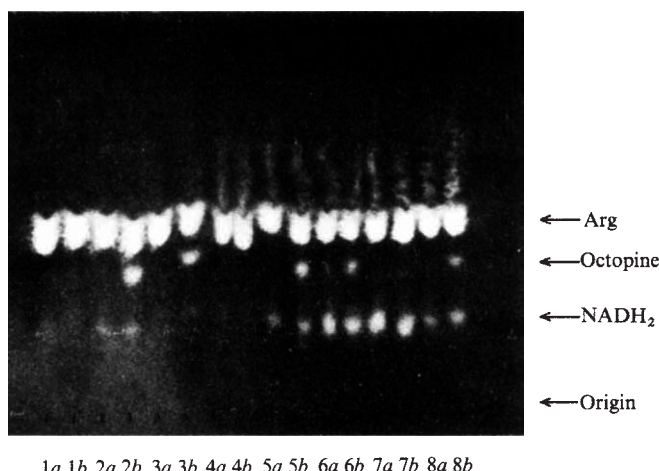
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## *In vitro* transformation of cultured cells from *Nicotiana tabacum* by *Agrobacterium tumefaciens*

CROWN GALL, the plant tumour of many dicotyledonous and some monocotyledonous species, is caused by *Agrobacterium tumefaciens* infection after wounding<sup>1</sup>. Crown gall cells, unlike cells derived from healthy plants, have the ability to proliferate indefinitely in culture without the growth hormones auxin and cytokinin<sup>2</sup>. The properties of the tumours, such as morphology (rough or smooth type) and the synthesis of the unusual amino-acid derivatives octopine or nopaline, are determined by the tumour-inducing (TI) plasmid present in the virulent bacterial strains<sup>3,4</sup>. The presence of at least a part of the plasmid has been demonstrated in the DNA of the tumour cultures<sup>4</sup>. Transcription of this fragment was also demonstrated in the cells of the



**Fig. 1** Electropherogram of the products present in the reaction mixture prepared to detect LpDH activity in extracts of different transformed calli and shoots. The enzyme activity was assayed according to Otten<sup>14</sup>. *a* Channels, reaction mixture at *t* = 0; *b* channels, reaction mixture at *t* = 60 min; 1, *N. tabacum* SR<sub>1</sub> shoot (control); 2, SR<sub>1</sub>-4013-1 shoot; 3, SR<sub>1</sub>-4013-3 shoot; 4, SR<sub>1</sub>-4013-28 shoot; 5, SR<sub>1</sub>-4013-1 callus; 6, SR<sub>1</sub>-4013-3 callus; 7, SR<sub>1</sub>-401328 callus; 8, B<sub>6</sub>S<sub>3</sub> crown gall tumour callus (control).

tumour tissues<sup>5,6</sup>. These findings seem to confirm the involvement of the TI plasmid in crown gall disease. However, it is not known if introduction of the TI plasmid alone into the plant cells is sufficient to obtain transformation of healthy plant cells into tumour cells. A fundamental approach to this question involves the development of an *in vitro* transformation system which can use TI plasmids of *A. tumefaciens* as vectors of genetic material in experiments to modify the genetic information of plants. We report here the transformation of tobacco cells by virulent strains of *A. tumefaciens*, the isolation and characterisation of

**Table 1** Number of colonies in hormone-free medium after incubation of SR<sub>1</sub> cells with different strains of *Agrobacterium*

| Bacterial strain | No. of plates seeded | No. of colonies | Frequency (approximate) |
|------------------|----------------------|-----------------|-------------------------|
| LBA 4001 (p)     | 4                    | 4               | $1 \times 10^{-4}$      |
| LBA 4013 (p)     | 9                    | 34              | $6 \times 10^{-4}$      |
| LBA 4011 (c)     | 6                    | —               | —                       |
| LBA 305 (p)      | 6                    | 1               | $1.5 \times 10^{-5}$    |
| LBA 301 (np)     | 6                    | —               | —                       |

To obtain SR<sub>1</sub> cells, protoplasts were isolated<sup>9</sup> from sterile shoots<sup>10</sup> and cultured in K<sub>3</sub> medium<sup>9,10</sup> supplemented with naphthalene acetic acid (0.1 mg l<sup>-1</sup>) and kinetin (0.2 mg l<sup>-1</sup>)<sup>12</sup>. The protoplasts were first kept in the dark for 24 h followed by 48 h at 2,000 lx. The cells were mixed with bacteria in a concentration of 10<sup>5</sup> cells per ml and 10<sup>7</sup> bacteria per ml. They were incubated at 20 °C for 32 h in K<sub>3</sub> medium. The light intensity during this incubation and all further treatments was 2,000 lx. After incubation free bacteria were removed by repeated washing and the tobacco cells (10<sup>4</sup> per ml) were cultured in K<sub>3</sub> medium supplemented with vancomycin (250 mg l<sup>-1</sup>), carbenicillin (200 mg l<sup>-1</sup>) and streptomycin (200 mg l<sup>-1</sup>). Three weeks later the colonies were collected by centrifugation, washed with hormone-free medium, and plated in Petri dishes (10 cm) into hormone-free K<sub>3</sub> medium solidified with 0.5% agar. The concentration was approximately 10<sup>4</sup> colonies per plate. This solid medium contained the same antibiotics as the previous liquid medium, plus erythromycin (200 mg l<sup>-1</sup>). Bacterial strains: LBA 4001, wild type *A. tumefaciens* Ach 5; LBA 4013, mutant of Ach 5 with a TI plasmid that is derepressed for transfer and constitutive for the octopine enzyme; LBA 4011, an avirulent derivative of Ach 5, cured of TI plasmid by growth at 37 °C, rifampicin resistant; LBA 301, wild type *A. radiobacter* S1005, non-pathogenic; LBA 305, S1005, converted to a pathogen by the introduction of a B6 octopine TI plasmid, p. Pathogen, carrying TI-plasmid; c, plasmid free, non-pathogen; np, non-pathogen.

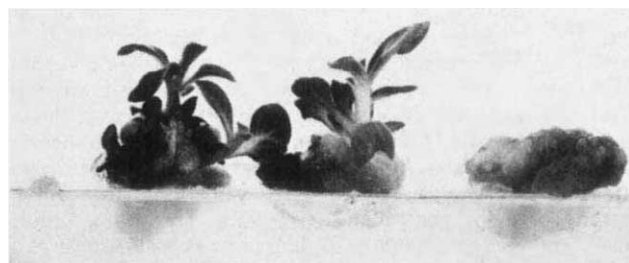
transformed calli and the regeneration of shoots with a distinct tumour marker.

Preliminary studies with mixed populations of healthy tobacco cells and B<sub>6</sub>S<sub>3</sub> crown gall tumour cells were carried out to define optimal conditions for recovery of transformed cells. These experiments were based on the properties of the tumour cells, such as hormone-independent growth and the synthesis of octopine, and on the properties of the *Nicotiana tabacum* SR<sub>1</sub> cells, such as hormone-dependent growth, streptomycin resistance and formation of green colonies in culture medium. It was found that protoplasts, cells or colonies of B<sub>6</sub>S<sub>3</sub> cells were able to grow in hormone-free medium whereas those obtained from the SR<sub>1</sub> plants could not grow in these conditions.

SR<sub>1</sub> protoplasts were isolated from leaves of sterile shoots and cultured for 3 d in medium with hormones. During this period the protoplasts regenerated new cell walls and a few underwent the first cell division.

The bacterial strains used were the virulent strains LBA 4013, LBA 4001 and LBA 305, and the non-virulent strains LBA 4011 and LBA 301; the strains are specified in the legend to Table 1. For transformation, the SR<sub>1</sub> cells were mixed with bacteria and incubated for 32 h. During this incubation, aggregation of cells and bacteria was observed. Cells aggregated within a few hours when LBA 4013 bacteria were present, whereas aggregation in the presence of LBA 4001 or LBA 4011 was observed only after at least 5 h. Incubation with LBA 305 and LBA 301 resulted in a slight aggregation that was observed only after 24 h. After incubation, the cells were washed and cultured in liquid medium supplemented with hormones, followed by subculture in solid medium without hormones. This selective medium allowed only hormone-independent cells to grow. Colonies, which were scored after a culture period of 7 weeks (Table 1), were only observed when SR<sub>1</sub> cells were incubated with virulent strains of *A. tumefaciens* and not in control plates when SR<sub>1</sub> cells were incubated with *A. tumefaciens* strains not containing the TI plasmid either after curing or as an original non-pathogenic isolate.

Colonies were isolated and subcultured for further growth and characterisation on selective and non-selective medium; their properties are listed in Table 2. Most of the SR<sub>1</sub>-4013 calli retained the ability to proliferate hormone independently after subsequent passages on hormone-free medium. Some of these calli demonstrated lysopine dehydrogenase (LpDH) activity whereas others did not. It is obvious that the group of calli which are hormone independent and LpDH positive is the most conspicuous, because it contains the highest number of calli in which the two tumour markers are observed. Another interesting callus is the SR<sub>1</sub>-4013-5, which apparently lost its ability to proliferate hormone independently even though LpDH activity was present.



**Fig. 2** Five-months-old differentiating SR<sub>1</sub>-4013-1 callus obtained after incubation of SR<sub>1</sub> cells with *A. tumefaciens* LBA 4013. The callus was isolated from K<sub>3</sub> medium without hormones and cultured for 6 passages in LS medium without hormones, solidified with 0.8% agar at 2,000 lx.

Before comparing the tumorous calli obtained from these *in vitro* transformation experiments with calli obtained *in vivo* after wounding and infection of plants with the same virulent bacterial strains, it is worth mentioning that the *in vivo* calli, which we have isolated with the LBA 4013, always contain both tumour markers. These calli have been cloned and subcultured for more than 2 yr during which time no reversion to hormone dependence or LpDH deficiency has been observed. This indicates that the loss of tumour markers in callus tissues is a specific phenomenon for those tumours obtained after *in vitro* transformation. Similar observations, concerning the separation of these tumour markers in callus cultures, have not been published before.

The most likely explanation for the observation that during subculture not all the hormone-independent calli contained LpDH activity and for the detection of enzyme activity in hormone-dependent calli is segregation of genes coding for these different properties of the tumour cells. This phenomenon is now being investigated by experiments involving the isolation of TI DNA from the calli and its characterisation using the Southern blot procedure<sup>8</sup>. A correlation between the presence of certain parts of the TI DNA and the expression of certain tumour markers has recently been found in *in vivo* transformation experiments by the isolation of virulent plasmid deletion mutants of *A. tumefaciens* which have lost the property to establish octopine synthesis in tumours which they induce<sup>15,16</sup>.

Although most calli were isolated after incubation with the LBA 4013 strain, Tables 1 and 2 show that four calli originated from the use of LBA 4001 and one callus from the LBA 305. From control experiments, in which the non-virulent strains LBA 4011 and LBA 301 were used, no colonies have been isolated (Table 1). The four SR<sub>1</sub>-4001 calli did not have LpDH activity; two of them even lost their hormone-independent

**Table 2** Characterisation of cell strains obtained after incubation of SR<sub>1</sub> cells with pathogenic strain of *Agrobacterium*

| Cell-bacterium combination | Callus no.        | Streptomycin resistance | Growth without hormones | LpDH activity | Regeneration |
|----------------------------|-------------------|-------------------------|-------------------------|---------------|--------------|
| SR <sub>1</sub> -4013      | 1; 3; 22          | +                       | +                       | +             | +            |
|                            | 4; 10; 13; 14; 15 |                         |                         |               |              |
|                            | 17; 18; 19; 20;   |                         |                         |               |              |
|                            | 21; 23; 24; 25;   | +                       | +                       | +             | -            |
|                            | 26; 29; 30; 31;   |                         |                         |               |              |
|                            | 33; 34            |                         |                         |               |              |
|                            | 11; 28            | +                       | +                       | -             | +            |
|                            | 6; 7; 32          | +                       | +                       | -             | -            |
|                            | 16                | -                       | +                       | -             | -            |
|                            | 5                 | +                       | -                       | +             | -            |
| SR <sub>1</sub> -4001      | 2; 8; 9; 12; 27   | +                       | -                       | -             | -            |
|                            | 1; 2              | +                       | +                       | -             | -            |
|                            | 3; 4              | +                       | -                       | -             | -            |
| SR <sub>1</sub> -305       | 1                 | +                       | +                       | -             | -            |

Colonies were isolated and subcultured in hormone-free LS medium<sup>13</sup>. Their streptomycin resistance was tested by culturing them in LS medium with hormones and streptomycin (1 mg ml<sup>-1</sup>). The media were solidified with 0.5% agar. The light intensity was 2,000 lx.



character. The SR<sub>1</sub>-305 callus seemed to be hormone independent and negative for LpDH activity. Calli which were both hormone dependent and LpDH negative were recovered after incubation with the LBA 4013 and the LBA 4001 bacterial strains. Although tumour markers can no longer be detected in these calli after four passages, they were isolated as hormone-independent colonies and obtained from the selective medium from those cultures of cells incubated with virulent bacterial strains.

Our results suggest that the calli in which tumour markers were no longer detectable after four passages have lost their transformed character by loss of the tumour-determining genes during subsequent cell divisions. The significance of these calli is also being studied.

The different numbers of transformed colonies observed by using the three virulent bacterial strains (Table 1) indicate a high efficiency of transformation by the LBA 4013, as compared with the LBA 4001 and the LBA 305 strains. This might be related to the ability of the LBA 4013 to transmit the TI plasmid to other bacteria in a high frequency<sup>15</sup>. Further experiments will show whether these differences are statistically significant.

The colour of the calli varied from white to green (results not shown), but a positive correlation between colour and the other phenotypic traits could not be established. The same is true of the capacity to produce shoots (Table 2). These arose on two LpDH-negative calli, whereas three LpDH-positive calli could also regenerate shoots. These shoots were also LpDH positive (Fig. 1). Figure 2 shows one of the LpDH-positive calli with the shoots. The morphology of the LpDH-positive shoots differs from normal SR<sub>1</sub> shoots in that the transformed shoots show an extensive lateral shoot formation and almost no size growth. The stems and the leaves are relatively thick, and there is almost no apical dominance. Roots cannot be induced. Regeneration in the way described for the *in vitro* transformed SR<sub>1</sub> calli has not been observed on tumorous calli obtained after *in vivo* transformation of shoots of *N. tabacum*.

Extension of the observations presented here, using purified TI-plasmid DNA as the transforming vehicle, is now being carried out. Preliminary results from these experiments indicate that TI DNA alone is sufficient for tumour induction in cultured plant cells. The successful transformation of individual plant cells by *A. tumefaciens*, its regeneration into shoots in which tumour markers are expressed, and the possibility of isolating new bacterial strains containing recombinant TI plasmids<sup>17</sup> provide new tools for the introduction of foreign genetic information into plant species.

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## Rat × rat hybrid myelomas and a monoclonal anti-Fd portion of mouse IgG

MONOCLONAL antibodies can be produced by cultures of permanent cell lines derived by fusion of suitable myelomas with spleen cells from immunised animals<sup>1</sup>. So far, mainly two mouse myelomas, X63-Ag8 and NSI/1-Ag4.1, have been used for this purpose<sup>1,2</sup>. When cells from immunised mice are used in the fusion, the mouse × mouse hybrid myelomas can be grown as tumours in appropriate mouse strains. We describe here the preparation and use of a new clone of a rat myeloma, which is suitable for the derivation of rat × rat hybrid myelomas producing specific rat antibodies. The spent medium of hybrid myeloma cultures usually contains 1-20 µg ml<sup>-1</sup> antibody. The serum and ascites of the tumour-bearing mice, with few exceptions, yield 1-20 mg ml<sup>-1</sup> of antibody, which is 1,000 times more concentrated and very convenient for larger preparations.

There are several reasons for preferring rats to mice for the production of monoclonal antibodies. The yield of serum and ascitic fluid is about 10 times better in tumour-bearing rats than mice, an overwhelming economic advantage in large scale production. Although there is a marginal increase in the generation time (10 weeks in the rat and 8 in the mouse) the rat litters are usually large. The derivation of hybrid myelomas using cells from immunised rats is a very successful procedure for deriving rat alloantibodies or anti-mouse xenoantibodies<sup>3,4</sup>, but because the derived lines are of mouse × rat origin the growth of tumours *in vivo* in either rats or mice requires rather elaborate procedures or special strains. Clearly, rat × rat fusion would be advantageous for making xenogeneic anti-mouse and allogeneic anti-rat antibodies, as well as others, where large scale production is anticipated. This requires a suitable parental rat myeloma line.

Myeloma tumours in the Lou strain of rats have been available for some time<sup>5</sup>. One of them, R210, has been grown in culture and an azaguanine resistant mutant (210.RCY3.Ag1) prepared. It secretes κ chains and was used in the first successful fusion between two myelomas<sup>6</sup>. When tested for its ability to fuse to normal or immunised rat spleens it gave disappointing results. It was twice subcloned in soft agar and the clone finally selected for further study was called Y3-Ag1.2.3. It gave no revertants when batches of 10<sup>7</sup> cells were tested. Fusion efficiencies were at first disappointing and the line was grown continuously in a spinner flask and tested for fusion efficiency at

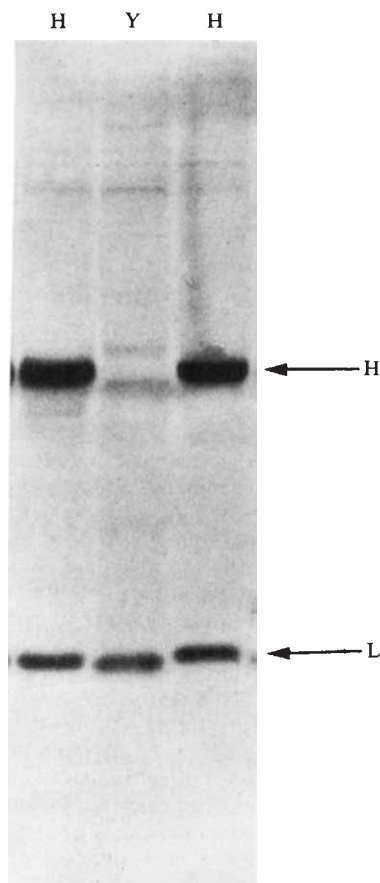
**Table 1** Species specificity of YA2/40

| Species    | Serum dilution giving<br>50% inhibition |
|------------|-----------------------------------------|
| Mouse      | 1:10,000                                |
| Guinea pig | 1:2,000                                 |
| Rabbit     | 1.40                                    |
| Horse      | >1:10*                                  |
| Human      | >1:10*                                  |
| Rat        | >1:0*                                   |

Inhibition of [<sup>3</sup>H]YA2/40 binding to Sp2 coated SRBC was as described in Fig. 2.

\* No inhibition detected at 1:10.





**Fig. 1** Analysis of Ig secreted by the rat hybrid myeloma clone YA2/40 H(LK). Cells were incubated for 24 h with  $^{14}\text{C}$ -lysine and supernatants were analysed<sup>2</sup> by SDS-polyacrylamide (10%) gel electrophoresis after total reduction. Y refers to Y3-Ag1.2.3 (control); H to Y2/40 H(LK) clone. DA rats were immunised by five footpad injections at 3-week intervals with 100  $\mu\text{g}$  of mouse IgG in complete Freund's adjuvant and boosted by intravenous injection without adjuvant 4 days before fusion. Supernatants were tested 13–15 days after fusion by indirect haemagglutination as follows:  $2.5 \times 10^8$  SRBC were coated by incubation for 1 h at room temperature with 5 ml of culture supernatants of anti-SRBC hybrid myelomas<sup>2</sup> minimally diluted to avoid agglutination. They were centrifuged and the pellet resuspended in 2.5 ml of phosphate-buffered saline (PBS). Aliquots of 25  $\mu\text{l}$  were distributed in V-bottom microtitre trays and 25  $\mu\text{l}$  of supernatant added to each well. After 1 h incubation the plate was centrifuged at 200g for 4 min and then kept in a 45° slope for 30–60 min. Culture YA2/40 was selected in this way and cloned twice in soft agar.

different times. The first successful fusion was obtained after 5 months of continuous growth. Using about  $5 \times 10^7$  Y3 cells and  $10^8$  spleen cells the fusion efficiency has improved to a stage where it is comparable to the mouse lines. The Y3 rat cells and their hybrid derivatives are much smaller in appearance than the mouse X63-Ag8 cells. Early stages of hybrid growth are less obvious under the microscope. Y3 cells grow very well on DMM supplemented with 5% or less fetal calf serum and the clonability in soft agar is very good. The line and the hybrids derived from it give rise to rather diffuse clones, instead of the tight ones given by mouse  $\times$  mouse hybrids.

Our first successful derivation of a rat monoclonal antibody by fusion of Y3-Ag1.2.3 was with spleen cells from a DA strain rat which had been hyperimmunised with mouse IgG (see Fig. 1 legend). The fusion protocol used polyethylene glycol 1500 as previously described<sup>3</sup>. The hybrid culture was divided into 48 subcultures. In this experiment only 6 showed hybrid growth. The spent medium of the hybrid cultures was tested by indirect haemagglutination of sheep red blood cells (SRBC) which had

been sensitised with different mouse monoclonal antibodies specific for SRBC<sup>2</sup>. One hybrid was clearly positive when the SRBC were coated with Sp3 (an IgG1) but negative when coated with Sp2 (an IgG2b) or Sp1 (IgM). The positive hybrid was subsequently cloned in soft agar and 8 individual clones were selected. All were found to be positive by the same test. The antibody secreted by each clone was internally labelled with  $^{14}\text{C}$ -lysine and analysed by SDS-polyacrylamide gel electrophoresis and isoelectric focusing (Fig. 1) (data not shown). All appeared to be identical, revealing a heavy chain band of about 50,000 MW and a single band present in the light chain zone. We were not able to demonstrate the presence of the two expected light chains (the myeloma parental and the antibody specific light chains). It is quite possible that both have very similar mobilities. Three of the above clones were cloned again to ensure purity and increase stability. One of these subclones was named YA2/40 H(LK) and used in all the experiments described below.

Binding of internally labelled [ $^3\text{H}$ -Lys]YA2/40 to cells coated with different classes of anti-SRBC antibody showed that YA2/40 recognised mouse IgG but not IgM. Binding to Sp1 (IgM) coated cells gave the same value as the background binding to uncoated cells. Binding to Sp3 (IgG1) and Sp2 (IgG2) coated cells was 4.9 and 35 times background respectively, a difference which correlates with the number of antigenic sites recognised by Sp2 and Sp3 (the number of antigenic sites recognised by Sp1 is intermediate between Sp2 and Sp3; C. D. Wilde, A. Williams and C. M., unpublished). The failure of YA2/40 to agglutinate Sp2 coated red cells although it agglutinated Sp3 coated cells (see above) remains unexplained.

Coating with Sp2 gives a sensitive procedure to assay for binding of [ $^3\text{H}$ ]YA2/40, and further specificity studies were done by inhibition of this binding. These studies confirmed that YA2/40 recognised individual proteins of both  $\gamma 1$  and  $\gamma 2$  subclasses of IgG (Fig. 2a). Inhibition by an IgM was at least 50-fold less effective. At high concentrations of protein some inhibition was observed, probably because of IgG impurities in the preparation. The antigenic determinant in the IgG1 is located in the Fd region for the following reasons. The inhibition of binding by MOPC 21 is equally effective with its variant IF1 (a mutant lacking the CH3 domain<sup>7</sup>) and with the F(ab')<sub>2</sub> fragment of MOPC 21 which lacks the CH2 and CH3 domains<sup>8</sup>. In both cases the inhibitory titre on a molar basis was similar to the intact protein and slightly higher on a per weight basis (Fig. 2b). On the other hand the IF2 mutant protein (which lacks the CH1 domain) was a very poor inhibitor. The residual inhibition is probably due to a trace of IgG present in the IF2 purified from serum of tumour-bearing mice which can be detected by SDS-polyacrylamide gel electrophoresis. No intact H-chain was present in the F(ab')<sub>2</sub> while a trace impurity of 50,000 MW component was detected in the IF2 preparation (data not shown).

As regards species specificity YA2/40 seems to be completely negative towards human, horse or rat, but shows some degree of cross-reaction to rabbit serum. On the other hand it is inhibited quite effectively by guinea pig serum. This pattern of cross-

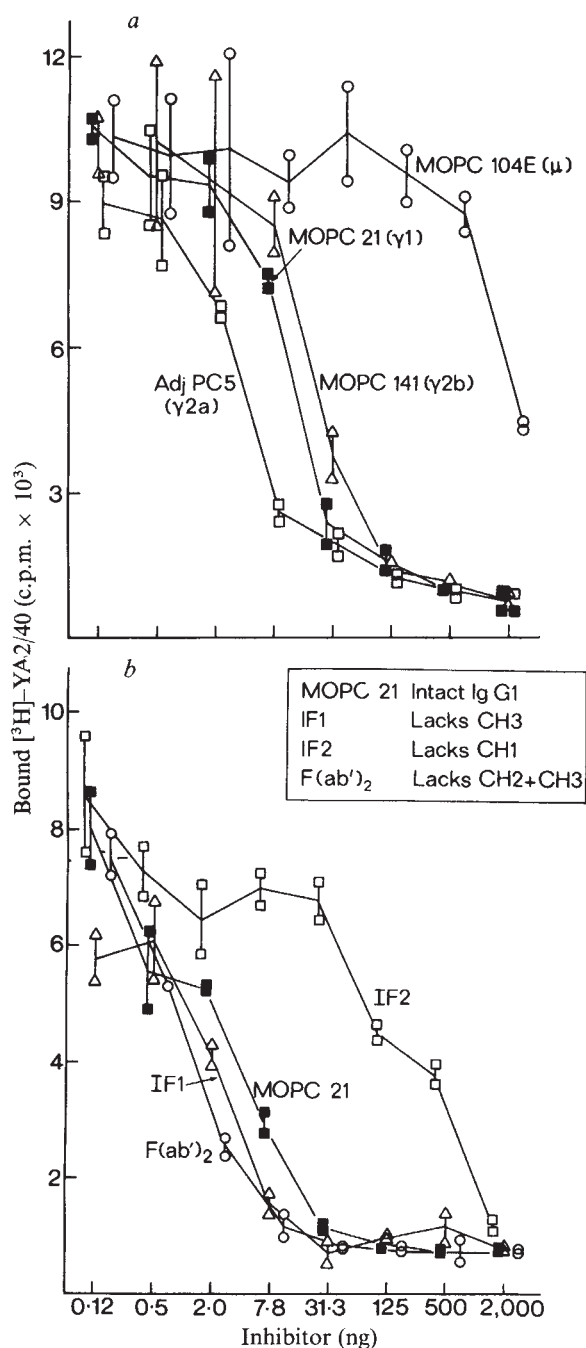
**Table 2** Preferential fixation of the hybrid immunoglobulin secretion phenotype following fusion with the rat myeloma line Y3-Ag1.2.3

|                                                         |    |
|---------------------------------------------------------|----|
| No. of cultures*                                        | 96 |
| Cultures showing growth of hybrids                      | 17 |
| Secretion of Y3-like light chain (only 12 tested)†      | 11 |
| Secretion of Ig chains different from Y3†               | 11 |
| Secretion of Y3 light chain in the absence of Ig chains | 1  |

\* Y3-Ag1.2.3 cells were fused to spleen cells from an immunised donor (DA rat immunised with cells from an AO rat). The culture was divided into 96 wells and allowed to grow for 4 weeks in selective medium.

† Tested by SDS-polyacrylamide gel electrophoresis of secreted products. Five of the cultures had insufficient numbers of cells for a meaningful test.

**Fig. 2** Inhibition of YA2/40 binding to IgG coated cells by other immunoglobulins.  $1.2 \times 10^6$  SRBC coated with Sp2/HL (IgG2b anti-SRBC) in 10  $\mu$ l of PBS were distributed in U-shaped microtitre plates. After addition of 20  $\mu$ l of inhibitor protein solution and 10  $\mu$ l of  $^3$ H-lysine-labelled YA2/40 (170,000 in a, 85,000 c.p.m. in b) and incubation at 4°C for 1 h, the cells were washed three times with cold PBS, the pellet lysed with 50  $\mu$ l of H<sub>2</sub>O and transferred to a scintillation vial. Counting was in 2 ml of scintillant (5 g PPO, 0.2 g POPOP, 540 g Triton X-100 and toluene to 1 l final volume). All proteins<sup>8</sup> were purified from serum by (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation and DEAE chromatography. F(ab')<sub>2</sub> fragment was prepared as before<sup>7</sup> and purified by Sephadex G-100 ultrafiltration. Internally labelled YA2/40 protein was prepared as follows:  $5 \times 10^6$  YA2/40 H(LK) cells were incubated in 5 ml of lysine-free medium containing 0.2 mCi ml<sup>-1</sup>  $^3$ H-lysine; after 8 h incubation at 37°C,  $5 \times 10^6$  more cells were added and the incubation continued for another 12 h. The supernatant was collected and dialysed three times against 0.5 l of PBS.



reactivity indicates that the antigenic determinant involves a similarity of local structures in guinea pig and the different mouse subclasses which, however, differ completely from the human, horse and rat. A close inspection of the sequences of the CH1 of mouse  $\gamma$ 1 (ref. 9), mouse  $\gamma$ 2a (ref. 10), guinea pig  $\gamma$ 2 (ref. 11), rabbit<sup>12</sup> and man  $\gamma$ 1 (ref. 13) reveals few examples of such a pattern of sequence similarity. The most interesting sequence involves residues 209 (Ser in mouse and guinea pig, Asn in human), 206 (Pro or Ala in mouse and guinea pig, Lys in human) and 204 (Ala in mouse and guinea pig, Asn in human). This is a cluster of residues in an exposed corner near the V-C domains boundary in the tertiary structure of the Fab<sup>14</sup>. It is important to keep in mind that structures in Fab other than CH1 could be involved in the antigenic recognition, the deletion of CH1 having a general effect on the tertiary structure of the Fab. Elucidation of the rat and horse sequences could throw further light on the precise nature of YA2/40 determinant. YA2/40 H(LK) cells grow as solid tumours in F1 (Lou  $\times$  DA) rats and the serum of the tumour-bearing rats contains 10–15 mg ml<sup>-1</sup> IgG as measured by radial immunodiffusion, giving rise to a prominent myeloma band component in cellulose acetate electrophoresis. The tumour could also be grown as an ascitic tumour. This was achieved by an intraperitoneal injection of 0.5 ml of pristane about 2 weeks before the intraperitoneal injection of  $5 \times 10^7$  cells (P. Wright, unpublished).

In a subsequent experiment, hybrids obtained from this line fused with spleen cells of an immunised rat were analysed by their ability to secrete Ig different from the myeloma parent. This was done by SDS-polyacrylamide gel electrophoretic analysis of the secreted products. Out of 12 cultures tested (all probably monoclonal) 11 secreted Ig chains and only 1 failed to secrete chains different from the myeloma parent. So, well over 60% of the tested cultures (and even of total number of growing cultures) secreted new immunoglobulin. This value is even higher than that obtained for the mouse system<sup>2</sup> and is in line with the hypothesis that the fusion between a myeloma cell and the heterogeneous population of cells present in the spleen is a highly selective event which favours the rescue of active antibody secreting phenotype. In a wider context it indicates that for the recovery of a transient differentiated function in the form of an immortal cell line, it is best to use a fusing parental cell which is phenotypically close to the normal phenotype one attempts to immortalise.

In conclusion, 210RCY3-Ag1.2.3 is a myeloma cell line suitable for the derivation of rat  $\times$  rat hybrid myelomas. This seems a better alternative than the mouse  $\times$  mouse hybrids for large scale production of monoclonal antibodies of potential general use in research, medicine and industry. The anti-mouse IgG monoclonal antibody described here is an example of such hybrids.

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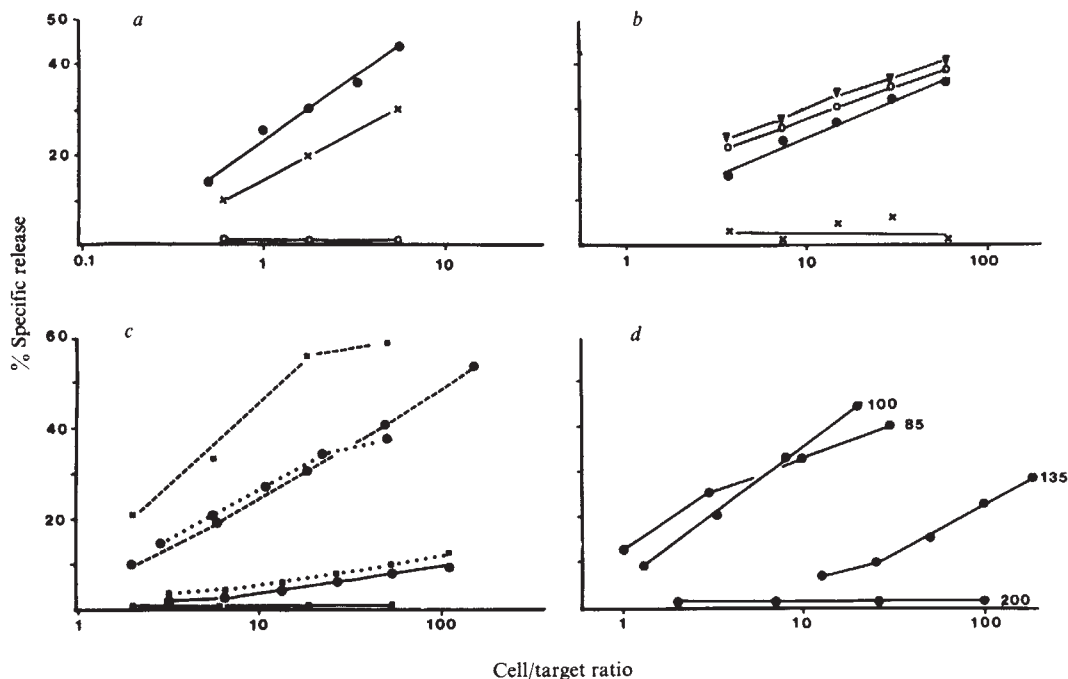
## Continuous cell lines with specific killer T-cell activity

THE establishment of continuous cell lines with effector T-cell activity is an obvious starting point in the isolation and characterisation of the T-cell receptor complex. Although nature provided the plasmacytomas which played a key part in elucidating B-cell immunoglobulin structure, there are no known examples of naturally occurring lymphomas with effector T-cell activity. Initial success with fusions between plasma cells and plasmacytomas encouraged extrapolation of this technique to T cells and thymic lymphomas<sup>1</sup>, but few promising results have so far been obtained. We have approached this problem by using the carcinogenic properties of mutagens to transform T lymphocytes responding in mixed lymphocyte culture (MLC), with the aim of isolating cell lines with killer T-cell activity. Here we report our success in the first step of isolating continuous, though antigen-dependent, killer T-cell lines.

We started with C.B20 (H-2<sup>d</sup> BALB/c, Ig-1<sup>b</sup> congenic) mouse spleen cells responding to irradiated (3,000 R) C57BL/6 (H-2<sup>b</sup>) spleen cells, in culture conditions described by McDonald<sup>2</sup>; 7 d after the second *in vitro* boost, cells were mutagenised (1 h) with (2 µg ml<sup>-1</sup>) nitrosoguanidine. Mutagenised cells were restimulated twice more, then seeded at 10, 10<sup>2</sup> or 10<sup>3</sup> cells per well in Falcon Micro Test II tissue culture plates and cultured with 10<sup>6</sup> irradiated C57BL/6 spleen cells per well which also served as a feeder layer. Individual wells were scored for growth, and killing capacity was tested by adding 5 × 10<sup>3</sup> of HAT (hypoxanthine, aminopterin, thymidine)-sensitive <sup>51</sup>Cr-labelled EL4.BU target cells (addition of HAT medium after assay killed all remaining EL4 cells leaving HAT-resistant MLC cells to grow). Although some wells had high killing activity, these wells

did not show good growth. There were 3 of 384 wells positive for growth from the 10-cell per well plates and 1 of 384 positive from the 10<sup>2</sup>-cell per well plates. The four isolates were expanded by weekly stimulation with irradiated C57BL/6 spleen cells and tested for growth requirements as well as killer activity. Each isolate contained only Thy 1.2-positive cells as tested by anti-Thy 1.2 and complement lysis (data not shown).

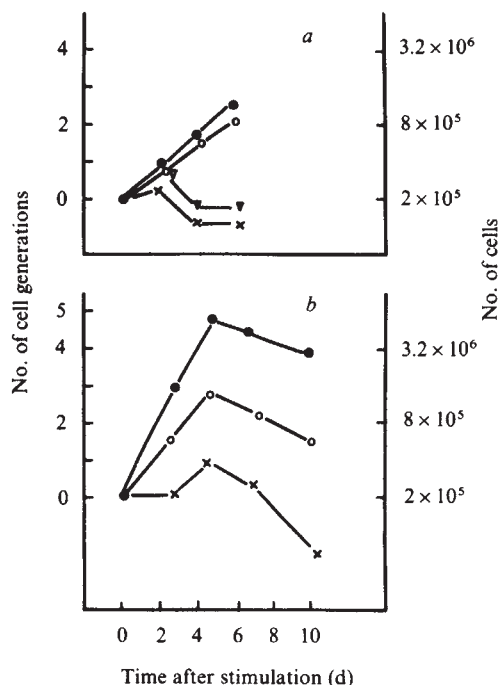
As shown in Fig. 1, the four isolates have different killer-cell activities. The isolates 100.1 and 10.3 show typical H-2D<sup>b</sup> and H-2K<sup>b</sup>-specific killing, respectively. Isolate 10.2 killed only in the presence of concanavalin A (Con A) or after Con A stimulation, and isolate 10.1 killed only EL4 and not C57BL/6 lipopolysaccharide blast targets. The specificity of killing exactly paralleled that of growth stimulation; for example, 10.3, which killed B10.A (5 R) (K<sup>b</sup>D<sup>d</sup>) but not B10.HTG (K<sup>d</sup>D<sup>b</sup>) was stimulated to grow with B10.A (5 R) but not B10.HTG. Isolates 10.2 and 10.1 were tested for growth with C57BL/6, B10.HTG and B10.A (5 R) irradiated spleen. The results show that 10.1 was specifically stimulated by B10.A (5 R) H-2K<sup>b</sup> (Fig. 2), although killing activity waned over the 150-d test period (Fig. 1). Isolate 10.2 showed a differential growth response, as C57BL/6 stimulated better than B10.A (5 R), and B10.HTG gave virtually no growth. The complex growth and killing properties of 10.2 have been studied in some detail without revealing any simple explanation; however, this isolate may well provide new insights on Con A-dependent killing. We found that 10.2, stimulated with Con A for 2 d then washed in medium containing α-methyl-D-mannoside, killed EL4 but not P815 (H-2<sup>d</sup>) targets. The isolate 10.2 could not be maintained in culture with either Con A or Con A-supernatant as stimulants, as indeed was true of all other isolates. The cytolytic efficiency of these different isolates varied from near normal MLC values (for example, 100.1) to zero (such as 150-d 10.1), suggesting that either the populations have different intrinsic killing efficiencies, or both active and inactive killers exist in the same population. When the isolates



**Fig. 1** Cytotoxic activities of four cloned isolates of T cells obtained from a C.B20 (H-2<sup>d</sup>) anti-C57BL/6 (H-2<sup>b</sup>) one-way MLC after multiple stimulation and mutagenesis. *a*, Isolate 100.1; *b*, isolate 10.3; *c*, isolate 10.2; *d*, isolate 10.1. Target cells labelled with <sup>51</sup>Cr were EL4 (●), P815 (□) and 2 µg ml<sup>-1</sup> Con-A blasted spleen cells from the congenic mouse strains C57BL/6 (H-2K<sup>b</sup>-D<sup>b</sup>) (▽), B10.A (5 R) (H-2K<sup>b</sup>-D<sup>d</sup>) (○) and B10.HTG (H-2K<sup>d</sup>-D<sup>b</sup>) (×). Cytotoxicity assays were carried out in flat-bottomed microtitre plates (Falcon 3040) in a total volume of 0.3 ml with 10<sup>4</sup> <sup>51</sup>Cr-labelled target cells and varying numbers of clone effector cells. The amount of <sup>51</sup>Cr released during 5 h incubation at 37°C was calculated on the basis of 0.1-ml samples and the per cent specific target cell lysis was calculated

according to the formula: [(experimental c.p.m. - spontaneous c.p.m.)/(total c.p.m. - spontaneous c.p.m.)]100. In experiments using tumour cell and Con A blast targets, spontaneous release was less than 10% and 25–35%, respectively. Isolate 10.2 (*c*) was assayed in the presence (---) or absence (—) of 10 µg ml<sup>-1</sup> Con A in the cytotoxic test. In one experiment 10.2 cells were cultured in the presence of 2 µg ml<sup>-1</sup> Con A for 48 h, then washed and incubated in 0.2 M α-methyl-D-mannoside for a further 2 h before testing in the cytotoxicity assay (.....). Isolate 10.1 (*d*) was assayed periodically over 154-d, the numbers indicating the time in days.





**Fig. 2** Isolates 10.1 (a) and 10.2 (b) were stimulated with 3,000 R irradiated spleen cells from C57BL/6 (●), B10.A (5 R) (○), B10.HTG (×) and C.B20 (▽). In all experiments cells were grown in Dulbecco modified Eagle's medium supplemented with 10% fetal calf serum,  $5 \times 10^{-5}$  2-mercaptoethanol,  $8 \times 10^{-2}$  M glutamine and non-essential amino acids. In the primary MLC,  $2.5 \times 10^{-7}$  C.B20 spleen cells were mixed with  $2.5 \times 10^7$  irradiated C57BL/6 spleen cells in 20 ml medium in Falcon flasks (3012) and incubated for 11 d in flasks standing upright. Subsequent stimulations, whether in flasks, Micro wells or Linbro wells were done at a concentration of about  $4 \times 10^4$  stimulator cells  $\text{mm}^{-2}$  tissue culture surface and 0.01 of the number of responder cells (except in the cloning procedure where the number of responding cells varied). All cultures were incubated at 37°C with an atmosphere of 15%  $\text{CO}_2$  in air.

are recloned it will be possible to distinguish these possibilities although our guess would be that low killing efficiency is more conducive to long-term survival of these cells in culture as exemplified by isolates 10.2 and 10.1.

Previous reports from other laboratories have shown that MLC cells can be maintained for long periods<sup>3,4</sup>, and in one case killer cells were 'cloned' in agar<sup>5</sup>. The use of soft agar to clone MLC cells was possible because cells could be maintained by Con A-supernatant stimulants, whereas in our case MLC cells require cellular stimulants and cannot be seeded in soft agar. The isolates we have obtained after growing MLC cells at limiting dilution are certainly clonal with respect to both the minimal growth unit and specificity; however, it is still impossible to conclude rigorously that a growth unit is in fact a single T-killer cell. Work is in progress to further mutagenise the isolates for the selection of antigen-independent killer T cells which can be formally cloned and studied as a homogeneous population of effector T cells. Recently, we have been able to use a more efficient carcinogenesis technique (developed by P. Patek and J. Collins, personal communication), which is based on continuous exposure of cultures to methylcholanthrene.

The ability to clone MLC responses seems to depend on prior mutagenesis, and with the techniques described here it is relatively straightforward to isolate growing clones of effector T killers. Further experiments are in progress to test if these killer-cell clones can act as abnormal suppressor cells in the system recently described by Epstein and Cohn<sup>6</sup>.

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## Effects of S-lactoylglutathione and inhibitors of glyoxalase I on histamine release from human leukocytes

THE enzyme responsible for the conversion of methylglyoxal to lactic acid by animal tissues was first named glyoxalase<sup>1,2</sup>. It was later shown that there are two glyoxalase enzymes<sup>3</sup>. The first (glyoxalase I) converts methylglyoxal and reduced glutathione to S-lactoylglutathione and the second (glyoxalase II) converts this compound to D-lactic acid, regenerating glutathione in the process. These enzymes are very widely distributed<sup>4</sup> and as yet have no clearly defined function(s). I describe here the enhancement of anti-IgE-induced histamine release by S-lactoylglutathione and its inhibition by an inhibitor of and an alternative substrate for glyoxalase I. These two compounds should, of course, decrease the production of endogenous S-lactoylglutathione. These experiments were undertaken for two reasons. First, a study from my laboratory has provided evidence that S-lactoylglutathione modulates microtubule assembly *in vitro*<sup>5</sup> while the release of histamine from leukocytes is a secretory process thought to require an intact microtubule system<sup>6,7</sup>. Second, concanavalin A has been shown to cause histamine release from human leukocytes<sup>8,9</sup> and has also been found to activate the two glyoxalase enzymes in lymphocytes and polymorphonuclear leukocytes<sup>10</sup>.

Leukocytes prepared from the blood of normal donors by dextran sedimentation as previously described<sup>11</sup> were subjected to hypotonic lysis (18 ml of ice-cold distilled water for 20 s followed by 2 ml of 9% NaCl) to remove contaminating red blood cells. They were incubated with anti-IgE and other compounds for 45 min at 37°C in a balanced salt solution consisting of (mM): NaCl, 110; KCl, 5.0;  $\text{CaCl}_2$ , 0.6;  $\text{MgCl}_2$ , 1.0; and PIPES buffer, 25 mM, adjusted to pH 7.3 with 40 mM sodium hydroxide. At the end of the incubation period the cells were centrifuged at 1,000g for 90 s and histamine released into the medium was determined using an automated extraction procedure<sup>12</sup>.

The effects of S-lactoylglutathione on histamine release induced by two different levels of anti-IgE from the cells of normal non-atopic donors are shown in Fig. 1. The dose-response curve to anti-IgE is bell-shaped<sup>13</sup> with maximum release varying from zero to approximately 60 or 70% depending on the individual from whom the cells are taken. The lower of the two anti-IgE concentrations studied is below that required for optimum release while the higher concentration is slightly above it. As can be seen, S-lactoylglutathione caused a small increase in release from the cells of 6 out of 7 donors at the lower anti-IgE concentration and from the cells of 9 out of 10 donors at the higher anti-IgE level. This enhancement is statistically significant with  $P < 0.003$  (Student's paired *t*-test) or  $P < 0.001$  (Wilcoxon matched-pairs signed-ranks test).

The effect of various concentrations of *S*-lactoylglutathione ( $0.2\text{--}2 \times 10^{-4}$  M) on histamine release were studied to establish the concentration necessary for optimum enhancement. The dose-response relationship varied with the cells of different individuals. With the cells of three out of five donors, maximum enhancement was seen at an *S*-lactoylglutathione concentration of  $2 \times 10^{-4}$  M. With the cells of one donor enhancement in the presence of  $5 \times 10^{-5}$  and  $2 \times 10^{-4}$  M *S*-lactoylglutathione was identical (51 to 59%) and with the fifth donor  $5 \times 10^{-5}$  M *S*-lactoylglutathione increased release from 60 to 73% while release in the presence of  $2 \times 10^{-4}$  M was only 64%. In the absence of anti-IgE, *S*-lactoylglutathione did not itself release histamine. As the preparation contained glutathione (Sigma) and this is one of the metabolic products of *S*-lactoylglutathione, the effect of glutathione ( $2.5 \times 10^{-4}$  M) was studied in seven experiments. It never enhanced histamine release and with the cells of two individuals somewhat (5–15%) inhibited release.

To investigate the role of *S*-lactoylglutathione in histamine release further, two structurally dissimilar compounds both capable of interacting with glyoxalase I and thereby decreasing *S*-lactoylglutathione formation were studied. The first of these, glutaryl-*S*-(*p*-bromobenzyl)-L-cysteinylglycine (PAL6-25-1) is an analogue of glutathione that is not metabolised by the enzymes capable of degrading glutathione<sup>14</sup>. It inhibits glyoxalase I from yeast with a  $K_i$  value of 0.32 mM, but apparently binds to a different site from that of glutathione<sup>15</sup>. The second compound, phenylglyoxal (Aldridge), is an analogue of methylglyoxal (Sigma) and is a substrate for glyoxalase I (ref. 16), forming *S*-mandelylgutathione rather than *S*-lactoylglutathione. *S*-Mandelylgutathione is a poor substrate for glyoxalase II, with a  $V_{\max}$  for this enzyme only a twentieth that of *S*-lactoylglutathione<sup>17</sup>. As can be seen from Table 1, both PAL6-25-1 and phenylglyoxal inhibited histamine release in a dose-dependent manner. Furthermore, the simultaneous addition of *S*-lactoylglutathione more than reversed the inhibitory effect of PAL6-25-1 and also partially (donor 1) or completely (donor 2) reversed the inhibition caused by phenylglyoxal. This reversal provides good evidence that PAL6-25-1 inhibits histamine release because it decreases endogenous *S*-lactoylglutathione formation. Phenylglyoxal is possibly acting in the same way although it is also conceivable that the *S*-mandelyl-

**Table 1** Effects of PAL6-25-1, phenylglyoxal and *S*-lactoylglutathione on histamine release from human leukocytes

| Inhibitor                             | Additions<br><i>S</i> -Lactoylglutathione<br>$2 \times 10^{-4}$ M | % Histamine release<br>(mean $\pm$ s.d.) |            |
|---------------------------------------|-------------------------------------------------------------------|------------------------------------------|------------|
|                                       |                                                                   | Donor 1                                  | Donor 2    |
| None                                  | —                                                                 | 58 $\pm$ 4                               | 64 $\pm$ 2 |
|                                       | +                                                                 | 71 $\pm$ 3                               | 60 $\pm$ 1 |
| PAL6-25-1<br>$3 \times 10^{-4}$ M     | —                                                                 | 45 $\pm$ 0                               | 51 $\pm$ 3 |
|                                       | +                                                                 | 76 $\pm$ 2                               | 66 $\pm$ 2 |
| PAL6-25-1<br>$10^{-3}$ M              | —                                                                 | 36 $\pm$ 4                               | 41 $\pm$ 0 |
|                                       | +                                                                 | 85 $\pm$ 3                               | 70 $\pm$ 2 |
| Phenylglyoxal<br>$10^{-5}$ M          | —                                                                 | 32 $\pm$ 1                               | 48 $\pm$ 2 |
|                                       | +                                                                 | 58 $\pm$ 4                               | 62 $\pm$ 2 |
| Phenylglyoxal<br>$3 \times 10^{-5}$ M | —                                                                 | 9 $\pm$ 2                                | 22 $\pm$ 1 |
|                                       | +                                                                 | 32 $\pm$ 2                               | 60 $\pm$ 2 |
| Phenylglyoxal<br>$10^{-4}$ M          | —                                                                 | 7 $\pm$ 1                                | 9 $\pm$ 2  |
|                                       | +                                                                 | 16 $\pm$ 3                               | 51 $\pm$ 4 |

Leukocytes from two normal individuals were incubated in triplicate as described in the text. The cells were preincubated for 5 min with PAL6-25-1, phenylglyoxal and/or *S*-lactoylglutathione after which anti-IgE (final concentration  $3.3 \times 10^{-2}$   $\mu\text{g ml}^{-1}$ ) was added and the incubation continued for 45 min. Donor 2 was specifically selected because previous experiments had shown that *S*-lactoylglutathione had little effect on anti-IgE-induced histamine release from her cells. *S*-Lactoylglutathione was prepared as described in Fig. 1.

glutathione formed from phenylglyoxal is an inhibitor of the action of *S*-lactoylglutathione.

The present findings implicate *S*-lactoylglutathione as a modulator of anti-IgE-induced histamine release and, as such, represent the first demonstration of an effect of this compound in any secretory system. In addition they are compatible with the general hypothesis that the function of the two glyoxalase enzymes is the regulation of *S*-lactoylglutathione levels. It is not yet known whether anti-IgE, the stimulus for histamine release, alters the activities of the glyoxalase enzymes and thereby increases *S*-lactoylglutathione levels in basophils. This is a definite possibility, however, as concanavalin A increases the activities of both glyoxalase enzymes in lymphocytes and polymorphonuclear leukocytes<sup>10</sup> and also causes histamine release from basophils through interaction with IgE<sup>9</sup>. The mechanism whereby *S*-lactoylglutathione potentiates histamine release is not clear. Because evidence has implicated *S*-lactoylglutathione in the process of microtubule assembly *in vitro*<sup>5</sup> while histamine release is a microtubule-dependent process<sup>6,7</sup>, it is possible that it affects histamine release, in part, by promoting microtubule assembly. It seems unlikely, however, that this is its only mode of action as disruption of microtubules with colchicine does not inhibit release to as large an extent as phenylglyoxal<sup>6,7</sup>. Elucidation of the precise role(s) of *S*-lactoylglutathione in histamine release will require further study.

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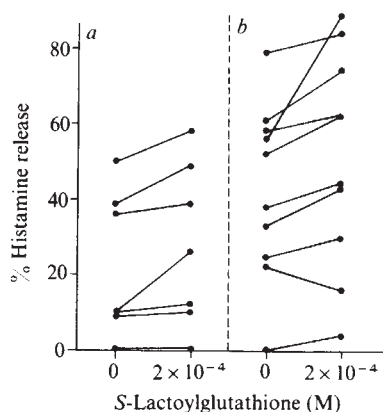
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**Fig. 1** Effect of *S*-lactoylglutathione on histamine release from human leukocytes induced by anti-IgE. Leukocytes from normal individuals were incubated in duplicate with anti-IgE (a,  $8.3 \times 10^{-3}$   $\mu\text{g ml}^{-1}$ ; b,  $0.167$   $\mu\text{g ml}^{-1}$ ) and with or without *S*-lactoylglutathione as described in the text. The release reaction was initiated by adding cells prewarmed to  $37^\circ\text{C}$  to tubes already containing all desired compounds. The average difference between duplicate measurements (higher value minus lower value divided by lower value  $\times 100$ ) was 3.8%. *S*-lactoylglutathione was prepared enzymatically by incubating a neutralised mixture of 20 mM methylglyoxal and 25 mM reduced glutathione with glyoxalase I (4 units  $\text{ml}^{-1}$ ) at room temperature for 1 h. In the presence of excess glutathione the conversion of methylglyoxal to *S*-lactoylglutathione was complete. The methylglyoxal was purified by steam distillation<sup>16</sup> followed by passage (at a concentration of 50 mM) over Dowex-1.



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## The influence of thymus on the development of MHC restrictions exhibited by T-helper cells

IMMUNE functions depend largely on the activities of peripheral lymphocytes which have been 'processed' in the thymus (T cells). The best characterised activities of these cells, such as *in vitro* cytotoxicity and suppressor and helper capacities in adoptive immunisations seem to be mediated by distinct subpopulations. However, it has emerged that T cells of all subpopulations exhibit a dual recognition faculty, on the one hand, towards entities coded by the major histocompatibility gene complex (MHC) and on the other towards foreign antigens. Without appropriate MHC recognition 'foreignness' may not be noticed, giving rise in various functional assays to what has been termed MHC restriction. For example, in the mouse, T cells may lack cytotoxicity unless they have H-2 region gene products<sup>1</sup>, in common with their target cells. T cells which 'help' in antibody responses can only do so fully if the B cells with which they are cooperating have similar I-region coding<sup>2</sup>. These various restrictions were thought to represent the need for interaction of like substances on cell surfaces perhaps to establish propinquity before any subsequent interactions could be initiated. Recently, however, it has been demonstrated<sup>3,4</sup> that irradiated bone marrow chimaeras of the type  $A + B \rightarrow (A \times B)F_1$ , possessed populations of T cells of *A* and *B* origin each of which was capable of interacting with B-cell populations of both *A* and *B* origin (*A* and *B* are used here purely as symbols for MHC-disparate mouse strains). Thus it seemed whatever the genetic determinacy of the cell, it could, when appropriately conditioned, establish reactions based on identity with either genetically similar or genetically different cells<sup>2,5</sup>. The exact nature of this conditioning began to emerge from experiments of Bevan<sup>6</sup> and Zinkernagel<sup>7</sup> who found that T cells from  $(A \times B)F_1 \rightarrow A$  chimaeras could only kill target cells bearing *A* type MHC determinants, whilst no cytotoxicity towards *B* targets was possible. Comparable studies gave analogous results for helper T cells<sup>8,9</sup> and recently, it was shown that the events which seem to determine the pattern of H-2 restricted specificities of the T cells concerned with cytotoxicity occur within the thymus gland<sup>7</sup>. We now show that intrathymic conditioning also determines the restriction specificities of T cells involved in helping antibody production.

The experimental design (Table 1 legend) aimed to generate populations of  $F_1$  T cells which had differentiated to their T-cell status within parental thymus grafts. In other studies it has been shown that both thymus lobes irradiated<sup>10</sup> as in the present series of experiments, and epithelial thymus grafts<sup>11</sup>, are incapable of generating T cells of the graft genotype but are capable of acting as staging houses for immigrant, unirradiated bone-marrow stem cells. From these and other studies it is a reasonable assumption that the majority of T cells in a thymectomised, irradiated, bone-marrow injected, thymus-grafted mouse such as those in the present studies, will have been processed by the (irradiated) thymus graft.

Thymic chimaeras were constructed using  $(C3H/He \times BALB/c)F_1$  recipients of irradiated thymus grafts. Three groups of experimental chimaeras were compared. One group received  $C3H/He$  ( $H-2^k$ ), another  $BALB/c$  ( $H-2^d$ ) and the third  $(C3H/He \times BALB/c)F_1$  thymus grafts. Helper function of spleen T cells from individual donors was then assessed on CBA ( $H-2^k$ ) and  $BALB/c$  ( $H-2^d$ ) B cells.  $(C3H/He \times BALB/c)F_1$  T cells which had matured through the influence of a  $C3H/He$  thymus tended to cooperate better with CBA B cells; those which had matured through a  $BALB/c$  thymus usually showed preference for cooperation with  $BALB/c$  B cells (Table 1). Those T cells which had differentiated through a  $(C3H/He \times BALB/c)F_1$  thymus were able to cooperate in all instances with both CBA and  $BALB/c$  B cells, perhaps with a slight bias towards CBA B cells. [Failure to cooperate efficiently with B cells bearing MHC specificities different to those of the thymus of maturation was unlikely to be the result of the 'wrong' haplotype markers on B cells eliciting nonspecific suppressors because in 10 thymic chimaeras tested, cooperation was always observed with  $(CBA \times BALB/c)F_1$  B cells whatever the restricted specificity of the helper population.]

**Table 1** Thymus determines MHC restricted specificities of T-helper cells

| Source of thymus graft      |            | Total no. of anti-TNP PFC with the following B-cells. |                 |
|-----------------------------|------------|-------------------------------------------------------|-----------------|
|                             |            | $CBA(H-2^k)$                                          | $BALB/c(H-2^d)$ |
| Expt 1*                     |            |                                                       |                 |
| $C3H/He$                    | Donor 1    | 1,676                                                 | 43              |
|                             | 2          | 1,902                                                 | 0               |
| $BALB/c$                    | Donor 1    | 367                                                   | 734             |
|                             | 2          | 996                                                   | 3,836           |
| $(C3H/He \times BALB/c)F_1$ | Donor 1    | 1,282                                                 | 1,823           |
|                             | 2          | 1,784                                                 | 1,068           |
| No T cells                  |            | 83                                                    | 52              |
| Expt 2*                     |            |                                                       |                 |
| $C3H/He$                    | Donor 3    | 315                                                   | 0               |
|                             | 3          | 77                                                    | 1,570           |
| $BALB/c$                    |            | 120                                                   | 97              |
| Expt 3                      |            |                                                       |                 |
| $C3H/He$                    | Donor 4    | 2,337                                                 | 205             |
|                             | 5          | 2,447                                                 | 207             |
| $BALB/c$                    | Donor 4    | 1,121                                                 | 1,943           |
|                             | 5          | 430                                                   | 419             |
| $(C3H/He \times BALB/c)F_1$ | Donor 3    | 1,804                                                 | 580             |
|                             | No T cells | 567                                                   | 30              |
| Expt 4                      |            |                                                       |                 |
| $C3H/He$                    | Donor 6    | 1,742                                                 | 320             |
|                             | 7          | 4,346                                                 | 321             |
| $BALB/c$                    | Donor 6    | 789                                                   | 1,681           |
|                             | Donor 4    | 2,047                                                 | 1,646           |
| $(C3H/He \times BALB/c)F_1$ |            | 324                                                   | 437             |
| No T cells                  |            |                                                       |                 |

Thymic chimaeras were constructed as follows:— $(C3H/He \times BALB/c)F_1$  mice were thymectomised at 6 weeks of age. One month after thymectomy the mice were irradiated with 875 R and reconstituted with  $(C3H/He \times BALB/c)F_1$  bone marrow (approximately  $5 \times 10^6$  bone-marrow cells per recipient) to produce relatively T cell-free ( $AT \times BM$ ) mice. Fifteen days later thymus lobes were obtained from newborn mice, irradiated *in vitro* with 600 R and transplanted under the kidney capsule of each  $AT \times BM$  recipient, one thymus lobe each. This procedure was designed to provide an environment for the maturation of host T cells without allowing any maturation of thymus-graft donor cells. Seven weeks later all recipients were primed with 250  $\mu$ g keyhole limpet haemocyanin (KLH) in Freund's complete adjuvant (FCA); after a further 3 weeks all mice were boosted with 100  $\mu$ g of soluble KLH and after 8–10 days the animals were killed and their spleens taken as a source of T-helper cells for *in vitro* experiments. T-helper function was assessed in an *in vitro* microculture system as previously described<sup>3,8</sup>. In brief, T cells were purified by passage of spleen cells through nylon wool. Red cells were removed by treatment with ammonium chloride and the remaining (T) cells treated at 37°C for 20 min with 25  $\mu$ g ml<sup>-1</sup> mitomycin C to eliminate the possibility of antibody production by any residual B cells. 'B cells' were spleen cells from TNP-OVA primed CBA or  $BALB/c$  mice, and were treated with anti-Thy 1.2 serum and complement. Helper T cells were incubated for 5 days with both  $BALB/c$  and CBA B cells in the presence of antigen (0.1–0.3  $\mu$ g ml<sup>-1</sup> TNP-KLH). Total direct and indirect PFC to TNP coupled donkey erythrocytes were then determined from 28–30 microculture wells.

\* The efficiencies of responses of  $BALB/c$  and CBA B-cells were compared in expts 1 and 2 by the addition of primed T cells of pools of  $(C3H/He \times BALB/c)F_1$  mice. In expt 1  $BALB/c$  B cells gave 2,818 PFC (in 30 wells) while CBA B cells gave 2,215 PFC. In expt 2  $BALB/c$  B cells gave 2,469 PFC while CBA B cells gave 1,831 PFC.



It is interesting that (CBA  $\times$  BALB/c) $F_1$  T cells which had matured in a BALB/c thymus should in most cases have cooperated with CBA B cells to some extent. This 'noise' in such MHC restriction experiments is not uncommon in our hands and was repeatedly observed in situations where BALB/c T cells, whatever the source, have been tested for helper function with CBA B cells. As suggested previously<sup>8</sup> this may represent an interesting one-way cross reaction between BALB/c and CBA MHC associated specificities.

This result is fully compatible with and clarifies previous studies with bone marrow chimaeric mice. Thus: (1) Totally allogeneic ( $A \rightarrow B$ ) chimaeras make poor antibody responses to thymus dependent antigens<sup>12</sup>. In this case A T cells develop in a B thymus and thus cooperate inefficiently with A B cells. (2) T cells from ( $A \times B$ ) $F_1 \rightarrow A$  chimaeras cooperate with A B cells only<sup>8,9</sup>, for they will have matured in an A thymus. (3) T cells from  $A + B \rightarrow (A \times B)$  $F_1$  double chimaeras cooperate with both A and B B cells<sup>3,4</sup> for they have developed in an ( $A \times B$ ) $F_1$  thymus. (4) T cells from  $A \rightarrow (A \times B)$  $F_1$  single chimaeras have the potential for cooperation with B B cells<sup>8</sup>, again as a result of developing in an ( $A \times B$ ) $F_1$  thymus.

It is also of interest that in the helper T-cell system it is the I region<sup>2</sup> which determines the observed MHC restrictions, for it is this region which also codes for MHC-linked Ir genes. It is possible to think that MHC restriction and MHC-linked Ir genes are but two faces of the same coin<sup>13</sup>, and indeed recent evidence lends support to this view, in studies where it was shown that MHC gene products (presumably in the thymus) can determine the T-cell repertoire to extrinsic (nominal) antigens<sup>14</sup>.

These results with helper T cells fulfil what could be predicted from the original findings of Zinkernagel *et al.*<sup>7</sup> with cytotoxic T cells; and reemphasise the point that MHC gene products are involved in the process of differentiation within the thymus.

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## Localisation of intestinal gastrin in a distinct endocrine cell type

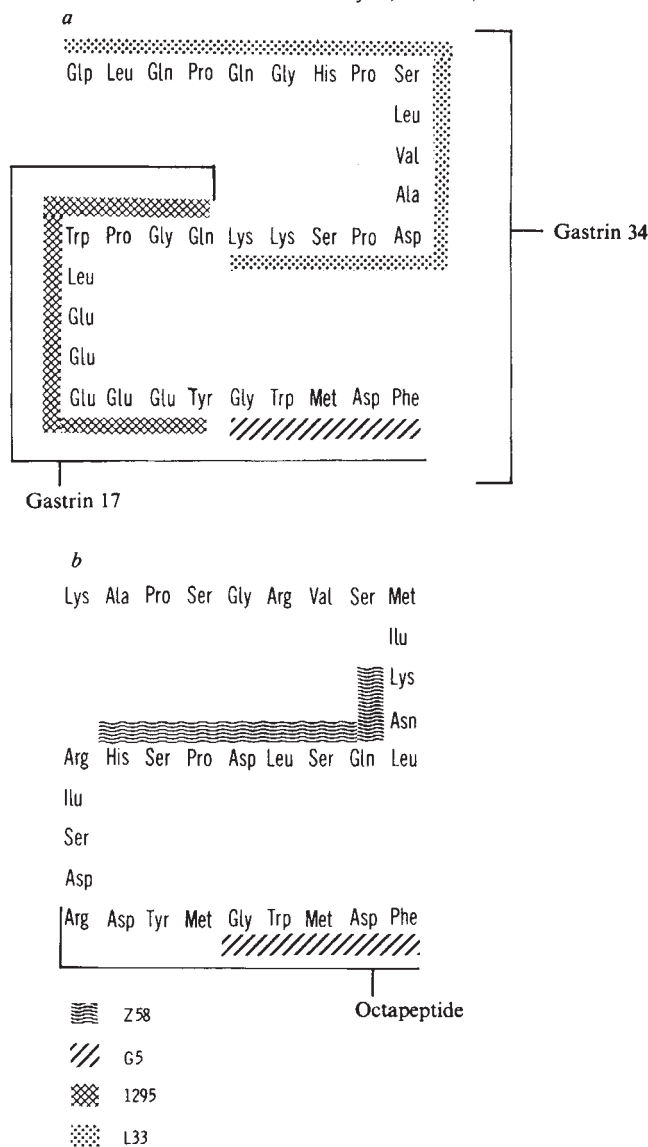
GASTRIN extracted from human intestine has two main molecular forms, little (G17) and big (G34) gastrins<sup>1</sup>. Both these peptides are also found in antral tissue although their relative proportions differ, G17 being the major component in antral tissue and G34 that in intestinal tissue<sup>1</sup>. The endocrine cell associated with the production of the immunoreactive gastrin found in the antral tissue is the ultrastructurally classified G cell<sup>2</sup>. The cellular origin of the immunoreactive gastrin extracted

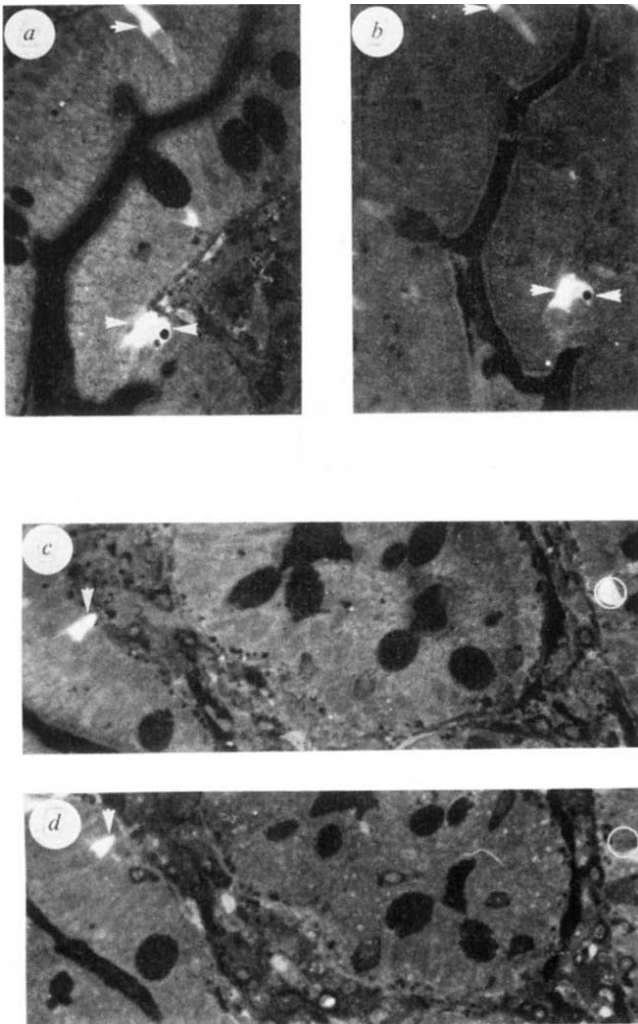
from the intestinal tissue has not been determined. The presence of cholecystokinin (CCK) peptides<sup>3,4</sup>, closely related to gastrin and sharing with it a C-terminal pentapeptide sequence (see Fig. 1), makes the specific immunological detection of both gastrin and CCK dependent on the use of well characterised and highly specific antibodies. We report here the finding that intestinal gastrin originates in a type of cell totally distinct from the I cell which has recently been identified as the source of CCK<sup>5</sup>.

To provide conclusive evidence we have used the semi-thin/thin sectioning method<sup>6</sup> with four different antisera. Each antiserum was specific for a different region of the peptide molecules, as shown in Fig. 1. G5 is a C-terminal-directed antiserum capable of detecting both gastrin and CCK<sup>7</sup>. 1295 is directed to the N-terminal region of G17 and detects principally little gastrin<sup>8</sup>. L33 is directed to the N-terminal region of big gastrin, G34, and detects the G34 form of gastrin<sup>9</sup>. Z58 was raised to a synthetic peptide with the 9-20 amino acid sequence of CCK; the antibodies detect only CCK from the series of homologous peptides (see Fig. 1). None of the antisera showed significant binding to any other available gut peptide, including secretin, vasoactive intestinal polypeptide, gastric inhibitory polypeptide, neurotensin, motilin, pancreatic polypeptide, somatostatin, glucagon, substance P and bombesin.

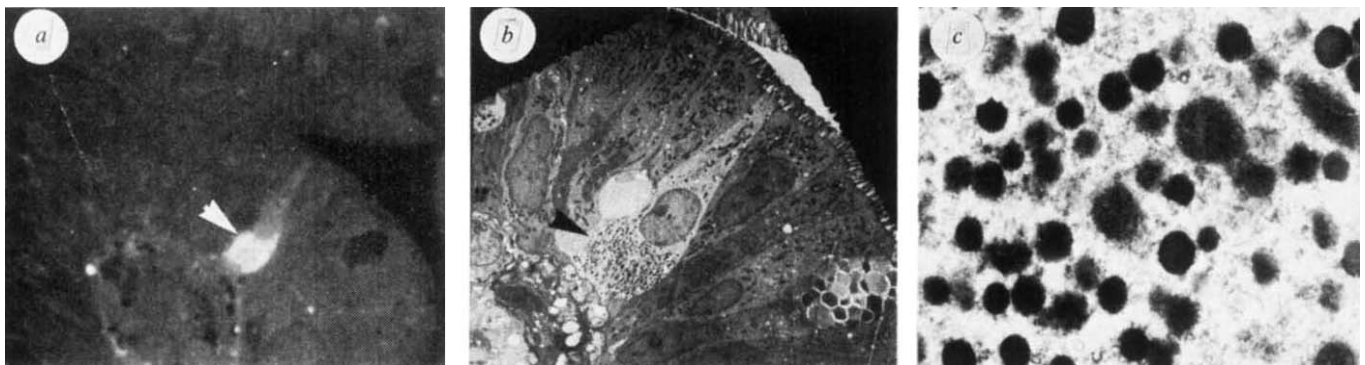
Semi-thin (800 nm) sections of human antrum, duodenum and jejunum, fixed in purified glutaraldehyde and embedded in resin, were stained by the indirect immunofluorescence

Fig. 1 Diagrams showing the region of the peptides detected by the antisera used in the study. a, Gastrin; b, CCK.





**Fig. 2** 800-nm (semi-thin) sections of human duodenum fixed in 2.5% purified glutaraldehyde. The araldite was removed by immersion in saturated NaOH in ethanol. The sections were stained by the indirect immunofluorescent technique using four region-specific antisera. *a*, Section stained with the 1295, N-terminal G17-specific antiserum, 1:300, for 24 h at 4 °C. Shows two positive cells, indicated by the arrows.  $\times 1,500$ . *b*, Serial section to *a* stained with the L33, N-terminal G34-specific antiserum, 1:100, for 24 h at 4 °C. Shows the same two positive cells.  $\times 1,500$ . *c*, Section stained with G5, the C-terminal gastrin/CCK antiserum, showing two positive cells made up of two separate populations: gastrin cells, marked by a circle; CCK cells, marked by an arrow (see *d*).  $\times 1,500$ . *d*, Serial section to *c* stained with Z58, the CCK-specific antiserum, showing the same arrowed cell as in *c*, positive, while the circled cell is negative.  $\times 1,500$ .



**Fig. 3** *a*, 800-nm section prepared as described in Fig. 2, stained with 1295 (1:300), the N-terminal G17 antiserum. The arrow indicates the positive endocrine cell.  $\times 1,500$ . *b*, Serial thin (60 nm) section showing the same endocrine cell. Note the connection to the gut lumen by microvilli and the grouping of the secretory granules close to the basement membrane.  $\times 2,000$ . *c*, High magnification electronmicrograph of the endocrine cell to show details of the secretory granules.  $\times 27,000$ .

method<sup>10</sup>. Serial sections were incubated with 1295 (1:300), G5 (1:100), L33 (1:100) and Z58 (1:200) overnight at 4 °C. A second layer of fluorescein isothiocyanate (FITC)-conjugated antirabbit globulin was applied for 1 h at room temperature. The specificity of the immunostaining was established by previous incubation with purified peptides as detailed in Table 1. The results of the tissue controls confirm the data already available on the specificity of the antisera. Additional controls were the incubation of serial semi-thin sections with normal rabbit serum (1:100) and FITC-conjugate alone, neither of which showed any positive cells.

The results of the immunostaining on the semi-thin (800 nm) sections of antral tissue showed that L33, 1295 and G5 all stained identical cells. These cells were then identified on the serial thin (60 nm) sections by means of easily located landmarks, such as mucin cells, which are visible in both semi-thin and thin sections. The ultrastructural appearance of the cells identified in this way was that of typical G cells with secretory granules ranging from large (350 nm) and electronlucent to small (190 nm) and electron dense. Z58 was negative on antral tissue as expected.

The semi-thin sections of duodenum and jejunum gave the following results. L33 and 1295 stained identical cells (Fig. 2*a*, *b*), Z58 stained a completely different population of cells (Fig. 2*d*), and G5 stained both cell populations (Fig. 2*c*).

As Z58 is specific for CCK and G5 is capable of binding to both CCK and gastrin, we conclude that the cells stained by both these antisera correspond to the I cells already characterised as the source of CCK<sup>5</sup>. This was confirmed by the appearance of the cells at an ultrastructural level. The cells stained by L33, 1295 and G5 must, by virtue of the specificity of the L33 and 1295 antisera, contain the immunoreactive gastrin present in the intestine. On serial thin sections these cells were found to be of a single type, with secretory granules which were small, round and electron dense (Fig. 3*a-c*). Electron micrographs of these cells were used to measure the secretory granules; 650 granules from 12 cells were measured, giving a mean diameter of  $175 \pm 24$  nm (mean  $\pm$  s.d.). When the measurements were corrected for sectioning artefact<sup>11</sup>, the final value for the mean diameter was  $220 \pm 20$  nm.

Clearly, the cells containing gastrin in the intestine are of a different morphological type from the G cells of the antral mucosa. They totally lack the large electronlucent granules that are so characteristic of the antral (GA) cells. Recent work has shown that the antral mucosa contains equimolar amounts of G17 and the N-terminal fragment left after cleavage of G34 to G17 (ref. 9), in addition to smaller quantities of G34 itself. It is possible that the large electronlucent granules in the antral (GA) cells contain the products of the enzymatic cleavage of G34, whereas the small granules present in the same cells contain mainly unaltered G34. Thus, the granules of the intestinal gastrin (GI) cells could correspond to these small G-cell granules



**Table 1** Specificity of immunostaining

| Antisera<br>(dilution) | Absorption | Peptides (nM) |            |                 |          | Area of gut |
|------------------------|------------|---------------|------------|-----------------|----------|-------------|
|                        |            | Gastrin 17    | Gastrin 34 | CCK octapeptide | CCK 1-33 |             |
|                        | Amount     | 0.3           | ND         | 1               | 2        | Antrum      |
| G5                     | Result     | —             | —          | —               | —        | Duodenum    |
| (1:100)                | Amount     | 1             | 5          | 10              | 10       | Jejunum     |
| 1295                   | Result     | —             | +++        | +++             | +++      | Antrum      |
| (1:300)                | Amount     | 10            | 2.5        | 10              | 10       | Duodenum    |
| L33                    | Result     | +++           | —          | +++             | +++      | Jejunum     |
| (1:100)                | Amount     | 10            | ND         | 10              | 1        | Antrum      |
| Z58                    | Result     | +++           | —          | +++             | —        | Duodenum    |
| (1:200)                | Result     | +++           | —          | +++             | —        | Jejunum     |

All absorptions were carried out by pre-incubation of the diluted antisera with the peptides overnight at 4 °C before staining the tissue. + + +, Positive immunostaining; —, no immunostaining; ND, not done; in this case the gastrin 34 peptide was only available in extremely small quantities, therefore only essential absorptions were undertaken.

and these may also contain mainly unaltered G34, which is the predominant molecular form of gastrin in the intestine.

Information from the study of tissue cultures carried out on gastrinoma cells<sup>12</sup> can be interpreted in the light of these findings. The cells in the original tumour contained the characteristic large electronlucent granules of G-cell type and the majority of the extracted gastrin was G17. After 2 weeks of culture the secretory granules had become progressively smaller and the content of the culture was predominantly G34.

The cells which we have identified as the origin of intestinal gastrin have probably been included in previous classifications as belonging to the ubiquitous D<sub>1</sub> cell category. These cells have been defined as containing small (140–190 nm) round granules, with moderate osmiophilia and a closely applied membrane. The retention of the D<sub>1</sub> nomenclature for the duodenal/jejunal cells containing gastrin would be confusing due to the widespread distribution of cells corresponding to the above description<sup>13</sup>. As the new variety of gastrin-containing cells is confined to the intestinal mucosa, we propose the name GI for these cells, denoting their intestinal origin, as distinct from the GA cells which are mostly confined to the antral region of the stomach. This is in keeping with the move towards a functional rather than morphological classification initiated in the Lausanne revision of the original Wiesbaden (1969) endocrine cell classification. Two new cell types have now been named after their peptide product, the N or neurotensin-containing cell<sup>13</sup> and the PP or pancreatic polypeptide-containing cell<sup>13</sup>.

In previous classifications a functional emphasis was not possible because there was insufficient evidence about the peptide products of the various cell types. The widely accepted semi-thin/thin technique, in combination with technical advances in antibody production and improved immunocytochemical methods have made a functional classification possible.

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## Identification of the neuritogen for experimental allergic neuritis

EXPERIMENTAL ALLERGIC NEURITIS (EAN) is a model of human acute inflammatory polyneuropathy, Guillain-Barré syndrome. It can be produced in several species of laboratory animal by immunisation with homologous or heterologous peripheral nerve emulsified in Freund's complete adjuvant<sup>1,2</sup>. After about two weeks the animals may develop signs varying from ataxia to paralysis and death. EAN has been produced using crude acid extracts of peripheral nerve<sup>3,4</sup>, but investigations into the neuritogenic properties of the component proteins of peripheral nerve myelin have yielded inconclusive results<sup>5–10</sup>. In experimental allergic encephalomyelitis (EAE), the central nervous system (CNS) counterpart of EAN, the encephalitogenic antigen has been shown to be a CNS myelin basic protein of molecular weight 18,000 (ref. 11). The three major proteins of peripheral nerve myelin, which together account for about 75% of the myelin protein, are usually referred to as P<sub>0</sub>, P<sub>1</sub> and P<sub>2</sub> (ref. 12). P<sub>0</sub> is a glycoprotein of molecular weight 30,000 and P<sub>1</sub> and P<sub>2</sub> are both basic proteins with molecular weights of 18,000 and 12,000–14,000, respectively<sup>12–13</sup>. We describe here a reliable method for producing severe EAN in inbred Lewis rats with bovine intradural root material, and show that intact P<sub>2</sub>, the major basic protein of peripheral nerve myelin, is the responsible neuritogen.

Bovine intradural root was obtained within 30 min of death and myelin was prepared from this by sucrose density centrifugation<sup>15</sup>. The crude acid extract was prepared by incubating myelin in 30 mM HCl for 16 h and collecting the supernatant



**Table 1** Production of EAN in Lewis rats with whole myelin and pure P<sub>2</sub> from bovine intradural root

| Antigen            | Dose<br>(mg per animal) | Day of<br>onset | Clinical disease |            | Histological disease |     | Incidence |
|--------------------|-------------------------|-----------------|------------------|------------|----------------------|-----|-----------|
|                    |                         |                 | Incidence        | Mean score | EAN                  | EAE |           |
| Saline             | —                       | —               | 0/2              | —          | 0/2                  | —   | 0/2       |
| FCA                | —                       | —               | 0/3              | —          | 0/3                  | —   | 0/3       |
| Myelin (5)         | 2.5                     | 12.0            | 25/25            | 2.5        | 24/24                | 2.6 | 0/24      |
| Acid extract (2)   | 0.25                    | 12.9            | 8/9              | 2.0        | 9/9                  | 2.4 | 0/9       |
| P <sub>2</sub> (2) | 0.25                    | 12.5            | 10/10            | 2.1        | 10/10                | 2.6 | 0/10      |

All samples except saline and adjuvant controls were emulsified in an equal volume of Freund's complete adjuvant (FCA) containing 10 mg ml<sup>-1</sup> *Mycobacterium tuberculosis* H37RA (Difco). The numbers in parentheses refer to the number of individual experiments carried out; a different antigen preparation was used for each. For scoring the clinical disease, the animals were assessed daily after day 7 according to the scale: 0 = normal; 1 = limp tail; 2 = mild or moderate paraparesis; 3 = severe paraparesis, tetraparesis or paraplegia. Scoring of histological disease were: 0 = normal or equivocal lesions, 1 = mild, 2 = moderate and 3 = severe, invasion of inflammatory cells.

after centrifugation. The basic protein, P<sub>2</sub>, was purified from this crude extract by ion exchange<sup>7</sup> and gel exclusion<sup>16</sup> chromatography. All steps were carried out at 4 °C in the presence of 1 mM mercaptoethanol; 5 g of lyophilised myelin yielded approximately 30 mg of P<sub>2</sub>. This was judged to be pure by the presence of a single band on SDS-polyacrylamide electrophoresis, similarity of amino acid content to that in previous reports (especially the absence of histidine)<sup>13,14</sup>, and the absence of ganglioside.

Male inbred Lewis rats (175–225 g) were given two subcutaneous injections of 50 µl emulsion, one just proximal to each fore-footpad, and were weighed and examined daily. All remaining animals were killed on day 21. Longitudinal sections of each sciatic nerve, the cervical and thoracic cord and brain, and six transverse sections of the lumbosacral cord and cauda equina from each animal were stained with haematoxylin and eosin and examined by light microscopy.

Preliminary experiments revealed bovine intradural root to be the best starting material and the inbred Lewis rat to be the most convenient species. In five separate experiments (Table 1), each with a different preparation of bovine intradural root myelin, moderate or severe clinical and histological EAN was always produced and the CNS showed no evidence of cellular infiltration. The lack of CNS involvement when using bovine peripheral myelin agrees with a recent report<sup>17</sup>. Clinical signs first appeared in some animals on day 11, with a mean onset of 12.0 d. This was accompanied by a weight loss to 75% of the starting weight.

The acid extract was then shown to be neuritogenic at a dose (0.25 mg per animal) which was calculated to contain a similar amount of P<sub>2</sub> as 2.5 mg whole myelin<sup>12</sup>. Finally, pure P<sub>2</sub> was shown to be neuritogenic at a dose of 0.25 mg. No CNS involvement was found in any of the animals treated with either the acid extract or pure P<sub>2</sub>.

Of the two basic proteins in peripheral myelin, P<sub>1</sub> has been shown to be the same as the encephalitogenic basic protein in the rabbit sciatic nerve<sup>18</sup>, and this is likely to be the same for other species. Thus, attention has focused on the other basic protein, P<sub>2</sub>, which may be specific to peripheral nerve myelin<sup>13</sup>. Previous attempts to induce EAN with a pure isolated protein have used bovine intradural root BF (same as P<sub>2</sub>) in the guinea pig<sup>6</sup>, bovine intradural root P<sub>2</sub> in the rabbit<sup>7</sup> and rabbit sciatic nerve P<sub>2</sub> in the monkey and guinea pig<sup>5</sup>. All but the last were unsuccessful, and this result was later withdrawn<sup>7</sup> with the suggestion that isolation might lead to an alteration in the considerable secondary structure of P<sub>2</sub>. In confirmation of this, Brostoff *et al.*<sup>10</sup> have reported that a cyanogen bromide peptide of P<sub>2</sub>, but not whole P<sub>2</sub>, was neuritogenic. In addition, Abramsky *et al.*<sup>8,9</sup> have isolated a basic protein of molecular weight 14,000 from human and bovine sciatic nerves which they reported to be neuritogenic. In the first report<sup>8</sup> this protein, known as P<sub>1</sub>L, was claimed to differ from all known myelin proteins, whereas in the latter<sup>9</sup> it was thought to be the same as P<sub>2</sub>. In both reports, however, the

disease produced was very mild, with an incidence as low as 50% in some experiments. Moreover, others have suggested that the reported amino acid content of P<sub>1</sub>L indicates contamination with other proteins, especially P<sub>1</sub> (ref. 14).

In the present study, a 100% incidence of pure EAN without EAE has been produced by the use of bovine intradural root P<sub>2</sub> in the inbred Lewis rat, a combination not previously reported. Thus, in contrast to the experience of others<sup>10</sup>, we have been able to isolate P<sub>2</sub> in a form which is still neuritogenic. Overall, the clinical disease produced by the pure neuritogen was slightly less severe than that produced by whole myelin, but, nevertheless, several animals developed the maximum score on our scale. A possible reason for the lack of success of previous authors, apart from the combination of tissue and recipient, may be their predominant use of the guinea pig<sup>4–9</sup>, because this species has been shown to possess very little P<sub>2</sub> in its peripheral myelin<sup>12</sup>. Thus, guinea pigs immunised with peripheral myelin basic proteins have been reported to remain unaffected<sup>6,7</sup>, develop EAE<sup>5</sup> or a low incidence of a very mild EAN<sup>9</sup>. In our preliminary investigation with the guinea pig, we found that bovine intradural root produced only EAE and not EAN.

Now that we have clearly identified the neuritogen involved in the animal model of Guillain-Barré syndrome, we intend to extend our study to look for evidence of sensitisation to P<sub>2</sub> in the human disease.

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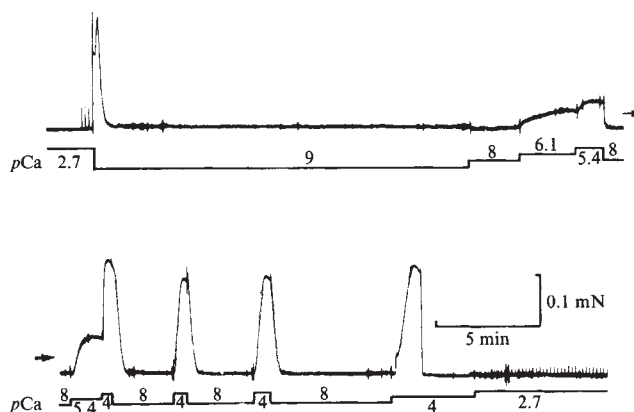
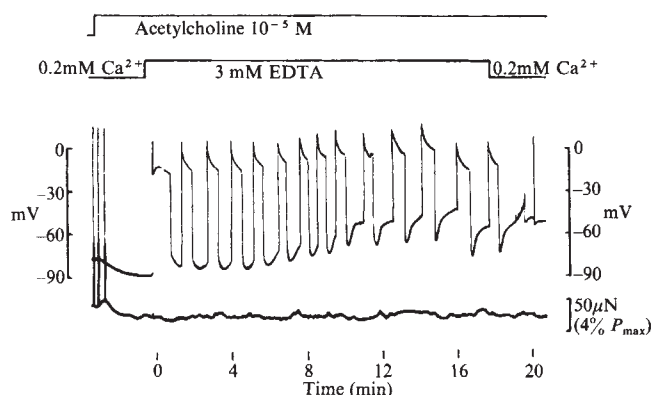
## Are cardiac muscle cells 'skinned' by EGTA or EDTA?

KNOWLEDGE of the processes involved in the contraction of skeletal muscle fibres has been greatly advanced by study of the 'skinned' fibre preparation. Here the sarcolemma is peeled off, giving access to the intracellular organelles unhampered by an intervening cell membrane. The small size of cardiac muscle cells had seemed to preclude the use of this technique, and so interest has been shown in a 'chemical' method for 'skinning' heart cells, which involves exposing the muscle to EGTA and EDTA (ref. 1). However, this paper questions the effectiveness of the EDTA/EGTA skinning methods, reporting that tension production in fully treated cells is  $[Na]_o$  dependent, and that the cells can support resting and action potentials in low  $[K]_o$ - 'skinning' media. It is concluded that both the high Ca-sensitivity and low membrane potential of EGTA/EDTA-treated cells occur without radical impairment of the sarcolemmal permeability barrier. An alternative explanation for the high sensitivity to extracellular calcium is presented which involves the Ca-Na exchange mechanism.

Treating cardiac muscle with divalent cation chelators such as EDTA and EGTA is reported to produce a 'high permeability state' after 12–20 min exposure to a medium which typically includes KCl, ATP and a buffer, in addition to the chelator<sup>1–6</sup>. The evidence that this procedure makes the heart cell membrane 'highly permeable to the calcium buffer system' (ref. 1) depends heavily on two principle observations: first, the treated cells have a very low resting potential, suggesting that the sarcolemma has become freely permeable to small ions<sup>1,4,5</sup>, and second, that tension can be induced by  $Ca^{2+}$  at micromolar levels. Mechanical activity at these low concentrations is normally associated with the activation of isolated contractile proteins or of skeletal fibres skinned by more direct methods<sup>1–6</sup>. The EDTA/EGTA method, or a variation of it, has subsequently been applied to several cardiac tissues, smooth muscle<sup>7</sup> and skeletal muscle<sup>8</sup>. However, recent studies of frog heart<sup>9,10</sup> have suggested that the two above characteristics are not unique to skinned cells and, therefore, are not unequivocally indicative of 'skinning'.

In these previous experiments, and those to be reported here, EGTA- and EDTA-containing solutions have been applied to fine atrial and ventricular trabeculae of frog heart by rapid

**Fig. 1** Electrical activity in EDTA-containing solutions. Membrane potential (upper trace) and tension (lower trace) from a superfused frog ventricle trabeculum (diameter  $\sim 200 \mu m$ , length 2 mm). On addition of acetylcholine ( $10^{-5} M$ ) to the Ringer solution (see ref. 9) stimulation was halted. Removing Ca and adding 3 mM EDTA induced spontaneous activity which persisted until EDTA was removed and Ca restored (after 17 min). A stimulus (at 20 min) produced an action potential of near-normal duration. Temperature  $20^\circ C$ .



**Fig. 2** Tension responses of a superfused frog atrial trabeculum (diameter  $\sim 100 \mu m$ , length 1.5 mm). Changing from Ringer's solution ( $pCa\ 2.7 = 2\ mM$ ) to Winegrad's 'disruption' medium ( $mM$ ; KCl 120,  $Na_2ATP\ 5$ , Tris 20, EDTA 5,  $pCa\ \sim 9$ ) induced a contracture.  $pCa$  values of less than 9 indicate that the 'activating' solution ( $mM$ ; KCl 120,  $Na_2ATP\ 5$ , Tris 20,  $MgCl_2\ 1$ , EGTA 5, Ca 0–5) was used. Halfway through the final exposure to  $pCa\ 4$ , the 'activating' solution was substituted by Ringer's solution at the same  $pCa$  value. Temperature  $20^\circ C$ ,  $pH\ 7.00$ .

superfusion. The results may be summarised thus. First, protracted action potentials are produced in EGTA-Ringer solution; these can arise spontaneously and eventually last up to a few minutes. Unless observed by continuous recording from one cell, this behaviour can give the appearance of a full depolarisation of the cell. However, even in preparations exposed to EGTA for over an hour the action potential can be curtailed by acetylcholine (ACh) or divalent cations<sup>9</sup>. The resting potential between the long action potentials is well maintained and, by inference, the cell membrane is still an effective permeability barrier. The effects also occur with EDTA and are more marked; spontaneous activity is common and action potential prolongation even greater. Figure 1 shows a typical record where the exposure to EDTA was started with the preparation already in the presence of ACh. ACh induces hyperpolarisation. Addition of EDTA rapidly evokes a spontaneous train of long action potentials which is sustained over 15 min. Restoration of  $Ca^{2+}$  returns electrical (and eventually mechanical) activity to near normal.

The second and, apparently for most authors it seems, the major criterion of the 'high-permeability state', namely high sensitivity to calcium, can also be displayed by cardiac cells without skinning them. Frog atrial trabeculae exposed to low sodium concentrations (with and without EGTA) produce half-maximal contracture tension at a  $[Ca^{2+}]$  of  $\sim 2 \times 10^{-7} M$  with a normal resting membrane potential<sup>10</sup>. This finding has been extended to frog ventricle where half-maximum tension in identical conditions occurs at  $1.2 (\pm s.d., 0.5) \times 10^{-6} M\ Ca^{2+}$  ( $n = 6$ ). Relaxation is very slow in these conditions ( $t_{1/2}$  of the order of 250 s at  $pCa\ 6$  compared with about 10 s at  $pCa\ 4-2$ )<sup>10,14</sup> and can give the appearance of a tonic rather than a phasic response. Until relaxation is completed, tension steps can be induced by  $pCa$  changes.

As both criteria for skinning fail to distinguish clearly between hypothetical skinned cells and those described above which are patently unskinned, it must be asked if cells exposed to the full EDTA and EGTA 'skinning' treatment are really skinned at all.

To identify whether the preparation undergoes a qualitative change in Winegrad's 'disruption' medium, the constituents of this medium were tested in various permutations. In summary, the high permeability state, judged on the development of high Ca sensitivity (repeatable step-wise responses to  $pCa$  changes), loss of membrane potential (except obviously in K-rich media) or production of rigor (high Ca, ATP-free media), could not be demonstrated even with the combination of EDTA or EGTA (up to 10 mM), high  $[K^+]$ , low  $[Na^+]$  and Tris buffer. However,



inclusion of ATP, which is present in Winegrad's full disruption medium, did produce a qualitative change in contractile behaviour.

If  $pCa$  steps were made after 20 min exposure of a frog ventricle or auricle trabeculum to EDTA or EGTA ( $pCa \geq 8$ , see legend to Fig. 2), then tension steps could be induced in the range of  $pCa$  7–4. However, there are at least four clear indications that these preparations are not skinned. (1) Spontaneous relaxation occurs, although at a very slow rate; (2) peak contracture tension at a given  $pCa$  falls with the duration of the exposure to the 'skinning' medium: the highest  $Ca$  sensitivity in these preparations develops within a minute but reaches a lower steady level after about 20 min; (3) rapid, full relaxation is produced when Ringer's solution is restored; (4) In an ATP + EGTA (or EDTA) Ringer solution ( $pCa > 8$ ), as before, long-lasting ACh-sensitive action potentials can be recorded for over an hour.

The third point is illustrated in Fig. 2. If these cells were in fact skinned, then restoring Ringer's solution (removing MgATP) while the muscle is contracted should, if anything, induce rigor<sup>11</sup>. Similar, though much slower, effects are visible in Winegrad's published records (ref. 1, Fig. 1). He interpreted this as due to a 'healing over' of the cell membrane at the raised calcium level. This argument can be discounted, as no relaxation occurs even at  $pCa < 2$  (see, for example, ref. 12, Figs 1, 2, 4) and relaxation occurs even without altering  $pCa$  (Fig. 2).

The relaxation produced by restoring Ringer's solution is primarily the result of raising the sodium concentration. This was shown by investigating the effect of  $[Na^+]$  on contracture strength in preparations that would otherwise be judged 'highly permeable'. Figure 3 shows the effect of  $[Na^+]$  from 6.6 to 63 mM on contracture strength at constant  $pCa$ . Three typical tension responses are shown on the right, from which it is clear that peak force is reduced as sodium concentration increases (note that  $[K^+]$  is constant and tonicity maintained with Tris). Results from one preparation are plotted on the left. The steep dependence of contractile strength at constant  $pCa$  on  $[Na^+]$  is not characteristic of skinned muscle but is reminiscent of responses to simple low-sodium media (ref. 10, Fig. 4). An expression derived in that paper (Eq. A6) relates contracture strength to  $[Na^+]_o$  at constant  $[Ca^{2+}]$ , and by choosing a suitable value for  $K_{Na}^{app}$  (which alters the position but not the shape of the curve) a good fit is obtained. The curve is shifted to the right on the Na axis at lower  $pCa$  values. This expression relates to the behaviour of a Na–Ca exchange system in the sarcolemma of frog heart. The results indicate that these supposedly skinned cells are not skinned at all, but rather that the tension responses observed result from the operation of the Ca–Na exchange system whose activity has been modified by depolarisation

(120 mM  $K^+$ ) and perhaps more importantly, by extracellular ATP.

Frog heart is not rendered 'highly permeable' by Winegrad's method. Whether a similar criticism may be levelled at results obtained with other cardiac and non-cardiac tissues using this technique<sup>2,4,7,8</sup> is open to speculation, although morphological studies of human skeletal muscle are reported to have shown disruption of the sarcolemma<sup>8</sup>. Long-lasting action potentials in EDTA have been reported for mammalian cardiac tissue<sup>13</sup>. The results reported here clearly indicate that the two properties of high calcium sensitivity and low resting potential are not sufficient indices of successful skinning. It seems that only the elegant and painstaking microdissection techniques of Fabiato and Fabiato<sup>15</sup> ensure that a cardiac cell can be skinned while leaving the sarcoplasmic reticulum and mitochondria intact.

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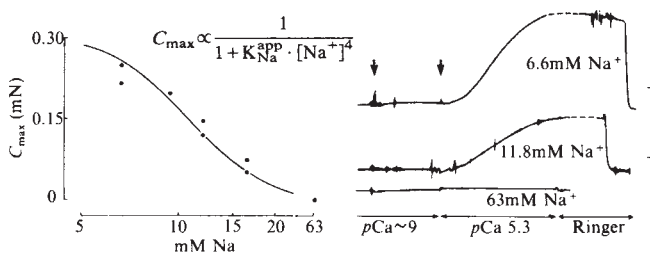
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## Activation of adenylate cyclase by vanadate

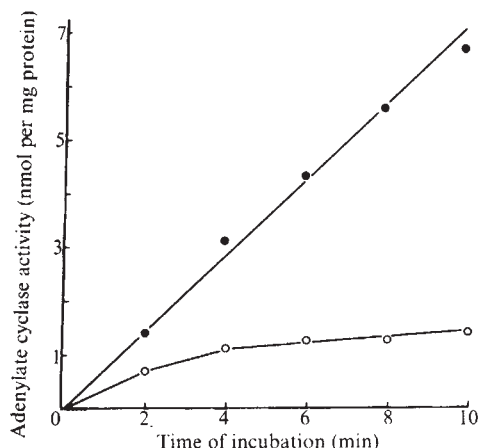
VANADATE has been identified as a potent inhibitor of  $(Na,K)ATPases$ <sup>1,2</sup>. The  $(Na,K)ATPase$  of dog kidney is inhibited 50% by 40 nM sodium orthovanadate ( $Na_2VO_4$ ) in optimal conditions (28 mM  $Mg^{2+}$ )<sup>2</sup>. As vanadate was originally identified as an inhibitor in muscle-derived ATP preparations<sup>2</sup> and in equine and rabbit skeletal muscles, it has been suggested as endogenous regulator of  $(Na,K)ATPase$ <sup>2–4</sup>. We have tried to use vanadate as a tool to inhibit ATPases in adenylate cyclase studies when working at low ATP concentrations. We report here that vanadate causes marked stimulation of adenylate cyclase activity in membranes isolated from rat fat cells and produces maximal activation even in the absence of hormones.

Plasma membranes of isolated fat cells were prepared from epididymal adipose tissue of male Sprague-Dawley rats (160–220 g). The preparation followed essentially the method of McKeel and Jarrett<sup>5</sup>. The determination of adenylate cyclase was carried out as described previously<sup>6</sup> using the protein-binding method of Gilman<sup>7</sup>. The assay contained in a total volume of 100  $\mu$ l: 1 mM ATP, 20 mM  $MgCl_2$ , 40 mM Tris-HCl, pH 7.4, 5 mM creatine phosphate, 10  $\mu$ g creatine kinase (25 units per mg) and 20  $\mu$ g bovine serum albumin. The reaction mixture was preincubated for 5 min at 37 °C and the reaction started by addition of freshly thawed plasma membranes (4–8  $\mu$ g per test tube). For blank values, heat-inactivated membranes were added to the reaction mixture. The reaction was stopped after 10 min incubation at 37 °C by addition of 1 ml of ice-cold



**Fig. 3** Right: tension records obtained after a 2-min exposure to the 'disruption' solution (see Fig. 2), at which time  $Ca$ -sensitivity is maximal. At the first arrow, the 'activating' solution was applied ( $pCa \sim 8$ ,  $Na_2ATP$  3.3 mM and Tris 127 mM otherwise as in Fig. 2, that is, hypertonic). At the second arrow, 1 min later, the  $pCa$  was reduced to 5.3 with  $[Na]_o$  varied by substituting NaCl for Tris-Cl. Finally, normal Ringer's was restored. Left: results from one experiment of the type shown on the right relating peak contracture tension ( $C_{max}$ ) to  $[Na^+]_o$ . The curve was drawn according to the equation and scaled to maximum possible tension taken as that observed in a 120 mM KCl, 0 mM NaCl solution<sup>11</sup>. The value of  $K_{Na}^{app}$  in this case was  $1.34 \times 10^8 M^{-4}$ . Calibration bar = 0.15 mN, temperature 20 °C, pH 7.00. (*Rana esculenta*.)



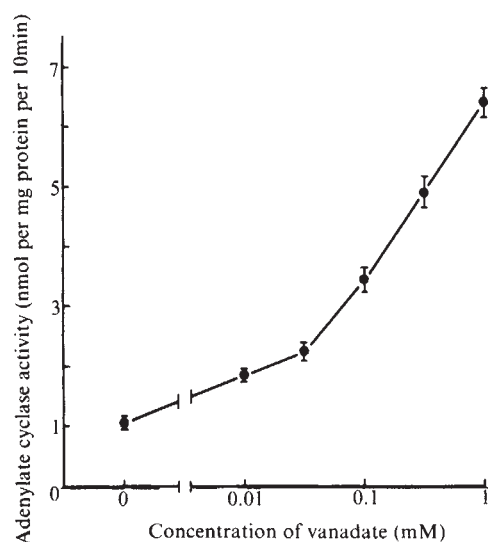


**Fig. 1** Time course of adenylate cyclase activation by vanadate. Purified plasma membranes were isolated from rat fat cells by a previously described method<sup>5</sup>. Fat cell membranes (4.4  $\mu$ g of membrane protein per 100  $\mu$ l) were incubated at 37°C in a medium containing 1 mM ATP with an ATP-regenerating system, 20 mM MgCl<sub>2</sub>, 0.2% bovine serum albumin and 40 mM Tris-HCl buffer, pH 7.4. Activity was assayed without (○) and with 1 mM sodium vanadate (●). Values are means of triplicate determinations.

50 mM sodium acetate buffer, pH 4.0. Cyclic AMP was determined after 60-fold dilution of the samples. In these conditions, vanadate up to a concentration of 1 mM did not interfere with the assay procedure. Compounds used were anhydrous sodium vanadate (NaVO<sub>3</sub>) and sodium fluoride (both Merck Darmstadt), theophylline (Boehringer Ingelheim), 1-noradrenaline-HCl (Fluka), guanylyl imidodiphosphate (GppNHp) and ATP (both Boehringer Mannheim).

Figure 1 shows a typical effect of sodium vanadate on the time course of adenylate cyclase activity in purified plasma membranes from rat fat cells. At a concentration of 1 mM, vanadate elicited an immediate stimulatory effect on enzyme activity which was linear up to 10 min. The increase of basal activity by vanadate was twofold after 2 min and more than fourfold after 10 min of incubation, the difference being due to a nonlinear time course of basal activity.

As can be seen from Fig. 2, the stimulatory effect of sodium vanadate was concentration dependent. A significant increase of



**Fig. 2** Concentration dependence of vanadate stimulation of fat cell adenylate cyclase activity. Fat cell membranes were incubated as described in the legend to Fig. 1. Values are means  $\pm$  s.e.m. of triplicate determinations.

cyclase activity was observed even with 10  $\mu$ M vanadate. Similar dose-response curves were obtained with ammonium vanadate (NH<sub>4</sub>VO<sub>3</sub>). Concentrations higher than 1 mM vanadate have not been tested.

The effect of vanadate on adenylate cyclase activity was further studied in the presence of a phosphodiesterase inhibitor and various stimulators of adenylate cyclase (Table 1). In the presence of theophylline, which itself had only a marginal effect on basal activity, the stimulatory effect of vanadate remained unchanged. Thus, it seems unlikely that vanadate might act as a phosphodiesterase inhibitor of a residual phosphodiesterase activity. Furthermore, vanadate in concentrations up to 1 mM had no effect on the activity of soluble and particulate cyclic AMP phosphodiesterases of rat fat cells (data not shown). The effect of 10  $\mu$ M noradrenaline was further increased by addition of vanadate, but the effects of the two agents were not additive. The activity obtained with NaF plus vanadate was somewhat lower than that with NaF alone. Vanadate increased the effect of a low concentration of GppNHp (0.1  $\mu$ M), but had no additional effect on a higher GppNHp concentration (1  $\mu$ M).

As it is a potent inhibitor of (Na,K)ATPase activity<sup>1,2</sup>, it seemed possible that vanadate might increase cyclase activity by preserving ATP as substrate for adenylate cyclase. However, cyclase activation by vanadate was independent of the concentration of ATP, tested in the range 1  $\mu$ M–1 mM (data not shown). In addition, the ATP-regenerating system used in the present study effectively preserved the ATP concentration even at concentrations lower than 1 mM (ref. 6).

**Table 1** Effect of vanadate on adenylate cyclase activity of rat fat cell membranes in the presence of theophylline or various stimulators of adenylate cyclase

| Additions                 | Control          | 1 mM vanadate    |
|---------------------------|------------------|------------------|
| None                      | 1.04 $\pm$ 0.04  | 6.43 $\pm$ 0.25  |
| Theophylline, 1 mM        | 1.20 $\pm$ 0.01  | 6.38 $\pm$ 0.22  |
| Noradrenaline, 10 $\mu$ M | 2.05 $\pm$ 0.03  | 6.77 $\pm$ 0.49  |
| NaF, 3 mM                 | 13.28 $\pm$ 0.76 | 11.89 $\pm$ 0.10 |
| GppNHp, 0.1 $\mu$ M       | 3.05 $\pm$ 0.24  | 5.89 $\pm$ 0.18  |
| GppNHp, 1 $\mu$ M         | 7.95 $\pm$ 0.16  | 8.13 $\pm$ 0.10  |

Fat cell membranes (4.4  $\mu$ g of membrane protein per 100  $\mu$ l) were incubated for 10 min at 37°C in a medium containing 1 mM ATP with an ATP-regenerating system, 20 mM MgCl<sub>2</sub>, 0.2% bovine serum albumin and 40 mM Tris-HCl buffer, pH 7.4. Each experiment was run in triplicate, values are means  $\pm$  s.e.m.

Another hypothetical explanation for a mechanism of action of vanadate might be that in addition to its inhibitory effect on (Na,K)ATPases, it may affect other nucleoside triphosphatases and cause activation of adenylate cyclase by inhibiting a GTPase associated with the guanylnucleotide binding protein. It has been suggested that the guanylnucleotide binding protein of the adenylate cyclase system plays a dual role<sup>8</sup>; in the presence of hormone plus GTP it activates the adenylate cyclase, but also functions in the hydrolysis of bound GTP and thus terminates the activation. The latter reaction has been referred to as the turn-off GTPase reaction<sup>8</sup>. Any decrease in the rate of hydrolysis of GTP at the regulatory site would maintain more of the enzyme at the active state. A reduction of GTP hydrolysis has been proposed as the basis for increased adenylate cyclase activity after cholera toxin treatment<sup>9</sup>. Furthermore an inhibition of catecholamine-stimulated GTPases by cholera toxin and an enhancement of GTP-dependent adenylate cyclase activity have recently been observed in turkey erythrocyte membranes<sup>8</sup>. Experiments are in progress to determine whether the adenylate cyclase activity elicited by vanadate is GTP dependent.

The effect of vanadate on cyclic AMP formation in addition to its effect on (Na,K)ATPases may be of general interest, as vanadate exerts biological activities in several intact cellular systems. A positive inotropic effect of vanadate in electrically driven cat papillary muscles<sup>10</sup>, and several pharmacological

effects, among them a powerful peripheral vasoconstriction, have been reported<sup>11</sup>.

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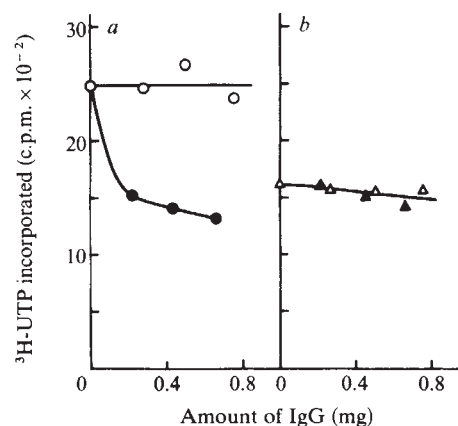
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## Antibody against a stimulatory factor of RNA polymerase II inhibits nuclear RNA synthesis

THE mechanisms by which eukaryotic genes are differentially activated are mostly unknown. However, the most fundamental process of gene expression is thought to be transcription by RNA polymerase II, and thus the regulation of RNA synthesis by this enzyme is one of the important points of study of eukaryotic gene expression. It is generally believed that structural modification of chromatin governs the transcriptability of the template<sup>1</sup>, and consequently studies have been carried out on the activity of chromatin<sup>2-4</sup>. Recently, another possibility has been considered, that various regulatory proteins may be involved in the process of transcription and regulate the gene expression<sup>5</sup>. However, although there are many reports of proteins stimulating the activity of RNA polymerase II *in vitro*<sup>6-11</sup>, no conclusive evidence has yet been obtained to show that these proteins are, in fact, functioning in the process of nuclear RNA synthesis. We have purified a protein from Ehrlich ascites tumour cells that specifically stimulates the activity of RNA polymerase II on homologous DNA<sup>12</sup>. Named S-II, this is a basic protein with a molecular weight of 40,500. We report here that an antibody against S-II markedly inhibits RNA synthesis in isolated nuclei, but that it does not affect the activity of purified RNA polymerase II. These findings suggest that the processes of RNA synthesis in isolated nuclei and with purified RNA polymerase II and deproteinised DNA are basically different, and that S-II has an essential role in nuclear RNA synthesis.

Protein factor S-II was extensively purified from Ehrlich ascites tumour cells, and antibody against S-II was prepared by injecting S-II into albino rabbits. This antibody was found to inhibit the stimulatory activity of S-II specifically. Nuclei were prepared from Ehrlich ascites tumour cells by the method of Marzluff *et al.*<sup>13</sup>, and were active in the incorporation of <sup>3</sup>H-UTP into RNA *in vitro*, in the presence of three other nucleoside triphosphates and salts.

First, we tested whether the antibody against S-II had any effect on RNA synthesis in the isolated nuclei. There are three species of RNA polymerase (I-III) in nuclei; RNA polymerases I and III, which synthesise ribosomal RNA and small molecular weight RNA, respectively, are fairly resistant to  $\alpha$ -amanitin, whereas RNA polymerase II, which synthesises messenger RNA, is inhibited by  $\alpha$ -amanitin<sup>14</sup>. Therefore, in tests on the effects of the antibody against S-II on the activities of these three

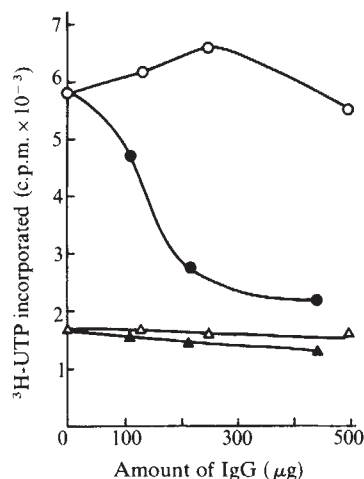


**Fig. 1** Inhibition of nuclear RNA synthesis *in vitro* by antibody against S-II. The reaction mixture contained, in a total volume of 100  $\mu$ l, 50 mM HEPES, pH 8.0, 0.25 mM each of ATP, GTP and CTP, 0.025 mM UTP, 2  $\mu$ Ci of <sup>3</sup>H-UTP (20 Ci mmol<sup>-1</sup>), 1 mM MnCl<sub>2</sub>, 5 mM Mg(OAc)<sub>2</sub>, 150 mM KCl, 12 mM  $\beta$ -mercaptoethanol, 0.05% Triton X-100, 10% glycerol, 0.5  $\mu$ g of  $\alpha$ -amanitin when present,  $2 \times 10^5$  nuclei and the indicated amount of antibody against S-II or normal IgG. The mixture was preincubated for 30 min at 0 °C, then at 25 °C for 10 min, after which acid-insoluble radioactivity was measured. *a*, Effect of the antibody on  $\alpha$ -amanitin-sensitive RNA synthesis; *b*, effect of antibody on  $\alpha$ -amanitin-resistant RNA synthesis. ●, ▲, Antibody; ○, △, normal IgG.

enzymes, assays were carried out in the presence and absence of 5  $\mu$ g ml<sup>-1</sup> of  $\alpha$ -amanitin. Nuclei were preincubated in the reaction mixture for RNA synthesis (see legend to Fig. 1) for 30 min at 0 °C with increasing amounts of antibody; the incubation temperature was then shifted to 25 °C and the incorporation of <sup>3</sup>H-UTP into the acid-insoluble fraction in 10 min was measured. The control contained immunoglobulin G prepared from non-immunised rabbit serum instead of antibody. As shown in Fig. 1*a*, the antibody inhibited nearly 50% of the  $\alpha$ -amanitin-sensitive RNA synthesis, whereas normal IgG had little effect. Neither antibody nor normal IgG affected RNA synthesis in the presence of  $\alpha$ -amanitin (Fig. 1*b*). These results clearly indicate that stimulatory factor S-II must be involved in some way in the RNA synthesis catalysed by RNA polymerase II in isolated nuclei, but that it is not related to  $\alpha$ -amanitin-resistant RNA synthesis. The results are consistent with our previous findings with isolated enzymes that S-II specifically stimulated RNA polymerase II and did not stimulate RNA polymerase I at all.

The question arises as to whether or not S-II is a component of the multimeric proteins of RNA polymerase II. We therefore tested the inhibitory effect of the antibody on purified RNA polymerase II. For this, we preincubated a fixed amount of purified RNA polymerase II with increasing amounts of the antibody at 0 °C for 30 min and then shifted the temperature to 37 °C and measured the incorporation of <sup>3</sup>H-UTP into RNA in 60 min. As a positive control, we measured the effect of the antibody on the stimulatory activity of S-II. As shown in Fig. 2, RNA polymerase II itself was not affected appreciably by either the antibody or normal IgG, whereas the stimulatory activity of S-II was specifically inhibited by the antibody but not by normal IgG. These results show that the antibody inhibits the activity of S-II. If S-II were an intrinsic component of multimeric proteins of RNA polymerase II, the antibody should have at least partly inhibited the activity of RNA polymerase II. It is possible that S-II is a component of native RNA polymerase II, but that it was split off during isolation of the enzyme, because when multimeric proteins are extensively purified their subunits are sometimes lost during the purification step, as in the case of  $\sigma$  factor of *Escherichia coli* RNA polymerase<sup>15</sup>. However, in this study we used partially purified RNA polymerase II (ref. 16), which was more likely to retain an intact molecular structure than completely purified enzyme. Thus, the fact that the antibody did





**Fig. 2** Effect of the antibody against S-II on partially purified RNA polymerase II. The reaction mixture contained in a total volume of 250  $\mu$ l, 40 mM Tris-HCl, pH 7.9, 3 mM  $MnCl_2$ , 4.6 mM  $MgCl_2$ , 50 mM  $(NH_4)_2SO_4$ , 0.068 mM EDTA, 4 mM  $\beta$ -mercaptoethanol, 0.25 mM each of ATP, GTP and CTP, 0.025 mM UTP, 0.5  $\mu$ Ci of  $^3H$ -UTP (20 Ci  $mmol^{-1}$ ), 5  $\mu$ g of Ehrlich ascites tumour DNA, 20 units of RNA polymerase II (see ref. 16), 25 units of S-II when present (see ref. 12) and the indicated amount of antibody against S-II or normal IgG. The mixture was preincubated for 30 min at 0°C, then at 37°C for 60 min, and then acid-insoluble radioactivity was measured. ●, Antibody + S-II; ○, normal IgG + S-II; ▼, antibody, no S-II; △, normal IgG, no S-II.

not inhibit the partially purified RNA polymerase II strongly suggests that S-II is not an intrinsic component of RNA polymerase II.

From the results obtained here using specific antibody against stimulatory factor S-II, two important conclusions could be drawn about eukaryotic transcription. First, in the process of transcription  $\alpha$ -amanitin-sensitive, nuclear RNA synthesis is not a simple process catalysed by RNA polymerase II, but in some way involves a regulatory protein(s) such as S-II. Second, the protein factors reported to stimulate RNA polymerase II *in vitro* are not necessarily components of the multimeric proteins of RNA polymerase II, but may exist as independent proteins that regulate transcription by interacting with the enzyme. Thus, in reconstruction of an accurate transcriptional system *in vitro* using purified RNA polymerase II and deproteinised DNA as template, attention must be paid to the effect of these stimulatory factors. This is the first report of evidence that a stimulatory factor is actually involved in nuclear RNA synthesis.

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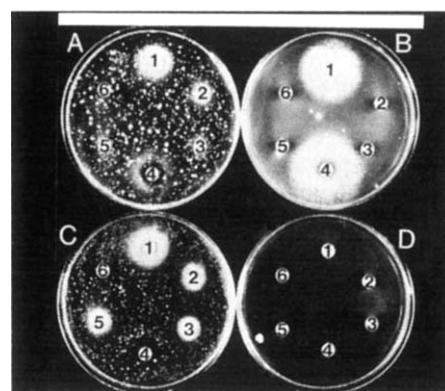
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## Phenotypic suppression and misreading in *Saccharomyces cerevisiae*

A NUMBER of antibiotics and other inhibitors have been useful in genetic and biochemical analyses of the protein-synthesising machinery of prokaryotic and eukaryotic organisms. Aminoglycoside antibiotics have been shown to be particularly helpful in this respect, especially in identifying ribosomal protein cistrons in bacteria. The aminoglycosides cause extensive misreading of the RNA code words *in vitro*<sup>1</sup> and suppress many nonsense and missense mutations in *E. coli*<sup>2</sup> phenotypically. The misreading observed in cell-free translation is believed to be the basis for the phenotypic suppression, although the exact mechanism is not known. Apart from a report of suppression of a single mutation in yeast by streptomycin<sup>3</sup>, there have been no demonstrations of phenotypic suppression in eukaryotic organisms. Earlier studies of mistranslation *in vitro* in eukaryotic systems indicated that this phenomenon is rare. Streptomycin did not cause misreading with cytoplasmic ribosomes of rat liver<sup>4</sup>, rabbit spleen<sup>5</sup>, chicken liver<sup>6</sup> or yeast<sup>7</sup>; neomycin had no effect in rabbit reticulocyte extracts<sup>4</sup> and only a slight effect in the yeast and chicken liver systems<sup>6,7</sup>. These results suggested that translation in higher organisms functions with higher fidelity than that in bacteria. Nevertheless, aminoglycoside antibiotics have recently been shown to cause extensive translational misreading *in vitro* in systems derived from *Tetrahymena*<sup>8</sup>, wheat embryo<sup>9</sup>, and cultured human cells<sup>10</sup>.

We report here that several aminoglycoside antibiotics (including hygromycin B, lividomycin, paromomycin and neomycin) cause phenotypic suppression of UAA (ochre), UAG (amber), and UGA mutations and also promote misreading *in vitro* in the simple eukaryotic organism *Saccharomyces cerevisiae*. Mutants resistant to hygromycin B have been isolated which exhibit altered levels of antibiotic-induced phenotypic suppression.



**Fig. 1** Test for phenotypic suppression of an UAA (A) and an UAG (B) mutation by various aminoglycoside antibiotics. Strain A carrying an ochre (UAA) mutation, *trp5-48*, and strain B carrying an amber (UAG) mutation, *met8-1*, were grown in yeast extract-peptone-dextrose medium to stationary phase. Cells were collected by centrifugation, washed twice with sterile water and resuspended in water. Approximately  $7.5 \times 10^6$  cells were spread on synthetic agar media. Sterile paper disks were placed as shown on which 500  $\mu$ g (25  $\mu$ l of 20 mg  $ml^{-1}$  aqueous solution) of the indicated antibiotics were dispensed. The antibiotics used were: 1, paromomycin; 2, lividomycin A; 3, gentamicin C<sub>1a</sub>; 4, hygromycin B; 5, kanamycin B; and 6, kanamycin C. Petri dishes were incubated at 30°C for 6 d and then photographed. The white growth surrounding a disk indicates phenotypic suppression. Strains C and D are mutants (see text) resistant to hygromycin B isolated from strains A and B, respectively. Tests with C and D were done exactly as described above for A and B.



Antibiotics were tested for phenotypic suppression of nonsense mutants of yeast as shown in Fig. 1; higher concentrations of antibiotics were also tested. Of 32 nonsense mutations tested, 18 were suppressible (Table 1) by one or more of the drugs. Only a few cases were found where a mutation not suppressed initially was suppressed when higher concentrations of antibiotic were used. The UAA mutation *trp5-48* and the UAG mutation *met8-1* were the most readily suppressible mutations of their respective classes. We therefore used these two mutations to test a variety of aminoglycoside antibiotics for their ability to cause phenotypic suppression (Table 2). Although paromomycin, hygromycin B and lividomycin B were generally the most efficient in causing phenotypic suppression, no clear-cut structure-activity relationship was evident. It has been reported<sup>8-10</sup> that only those aminoglycoside antibiotics that contain a 6'-hydroxyl group on the amino sugar linked to the 4-position of 2-deoxystreptamine were highly active in promoting mistranslation in eukaryotic cell-free systems. If suppression of auxotrophic mutations by the antibiotics in yeast is due to mistranslation, this structure-function relationship does not always apply. For example, in Fig. 1 (A, C) it can be seen that kanamycin B is substantially more active than kanamycin C; the reverse is predicted from *in vitro* mistranslation studies. In addition, several of the gentamicins were active.

**Table 1** Nonsense mutations tested for phenotypic suppression

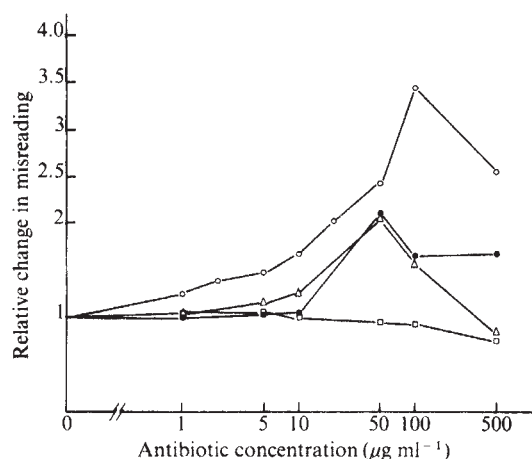
| Mutation type | Suppressed*                                                                                                               | Not suppressed                                                                             |
|---------------|---------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------|
| UAA           | <i>trp5-48, lys1-1, leu2-1</i><br><i>met15-30, met15-49,</i><br><i>met15-85, met15-95,</i><br><i>met15-119, met15-131</i> | <i>his5-2, ade2-1, arg4-17</i><br><i>can1-100, met15-47,</i><br><i>met15-70, met15-107</i> |
| UAG           | <i>met8-1, ilv1-1, aro7-1</i><br><i>ade3-26, met15-44</i><br><i>met15-46, met15-72,</i><br><i>met15-84</i>                | <i>trp1-1, met15-21,</i><br><i>met15-76</i>                                                |
| UGA           | <i>leu2-2</i>                                                                                                             | <i>his4-166, his4-260</i>                                                                  |

\* Suppressible by one or more antibiotics listed in Table 2.

Although most of the nonsense mutations were suppressed by one or more of the antibiotics, phenotypic suppression of missense mutants was rare. No auxotrophic mutations in yeast are known to be missense on the basis of amino acid substitution or nucleotide sequence data. A number of osmotic-remedial and/or temperature-sensitive mutants are, however, believed to be missense. Paromomycin, which is active on all phenotypically suppressible nonsense mutants (Table 1) was tested for suppression of isogenic series of missense mutants in the *met15* locus. Only one (*met15-33*) of 34 could be weakly suppressed. This mutant is osmotic-remedial, shows interallelic complementation and was not suppressible by amber and ochre suppressors<sup>11</sup>. The proportion of missense mutations that are suppressible in bacteria is lower than that of nonsense mutations<sup>12</sup>, but it seems to be much higher than in yeast. The frequency of phenotypically suppressible mutants in a random sample of mutants was relatively low; we found only ten such mutants in a collection of 337 independent auxotrophic mutants isolated from a prototrophic strain.

Some of the ochre (UAA) mutants that we examined contain the cytoplasmic determinant  $\psi$ , which increases efficiencies of ochre<sup>13,14</sup> and some frameshift<sup>15</sup> suppressors but has no effect on amber suppressors<sup>13</sup>; ochre mutations were more readily suppressed by antibiotics in  $\psi^+$  strains than in  $\psi^-$  strains. Since all the amber mutants were  $\psi^-$ , and the  $\psi$  state of UGA mutants was not determined, we do not know if the  $\psi^+$  genetic determinant facilitates phenotypic suppression of UAG and UGA mutants.

Of the aminoglycosides listed in Table 2, only hygromycin B (ref. 16) and destomycin A (A. S., unpublished) inhibit the growth of *S. cerevisiae* on glucose nutrient medium. These are structurally similar, destomic acid-containing antibiotics. We have selected a large number of mutants resistant to hygromycin B; they are recessive and represent at least three complementation groups. The mutants exhibited a wide variation in the level of phenotypic suppression, ranging from the wild-type response to complete restriction. Two examples are shown in Fig. 1. The resistant mutant (C) isolated from the *trp5-48* strain



**Fig. 2** Effect of aminoglycoside antibiotics on misreading in poly(U)-directed yeast cell-free protein synthesis. The 80S ribosomes and supernatant factors were prepared from wild-type yeast as described earlier<sup>17</sup>. The reaction mixture for poly(U)-directed incorporation of phenylalanine and leucine contained, in 50 µl: 50 mM Tris-hydrochloride, pH 7.7, 12.5 mM magnesium acetate, 80 mM KCl, 4 mM creatine phosphate, 40 µg creatine phosphokinase ml<sup>-1</sup>, 250 µg yeast tRNA ml<sup>-1</sup>, 1 mM dithiothreitol, 2 mM adenosine 5'-triphosphate, 0.1 mM guanosine 5'-triphosphate, 2 µM <sup>14</sup>C-phenylalanine (specific activity 486 mCi mmol<sup>-1</sup>), 2 µM <sup>3</sup>H-leucine (specific activity 8.8 Ci mmol<sup>-1</sup>), 300 µg poly(U) per ml, 1 mg ribosomes per ml, 2 mg supernatant enzymes per ml. Reactions were carried out at 30 °C for 45 min. 50 µl of 1 M NaOH was added and the mixture was incubated for an additional 20 min. After addition of one ml of 10% trichloroacetic acid the resulting precipitate was collected on glass-fibre filters (Whatman GF/C), washed with 5% trichloroacetic acid and dried. Radioactivity was determined in a toluene-based liquid scintillation solution. Absolute amounts of poly(U)-directed incorporation of phenylalanine and leucine were determined. The data are presented as ratios of misreading observed in the presence of the antibiotics to that found in their absence. As a typical value, the ratio of 1 corresponds to misreading (Leu/Phe ratio) of  $4 \times 10^{-3}$  which in turn corresponds to the incorporation of 50 pmol phenylalanine and 0.2 pmol leucine. ○, Hygromycin B; ●, lividomycin B; △, paromomycin; □, streptomycin.

(A) had a reduced level of suppression by hygromycin B. On the other hand, the hygromycin B-resistant mutant (D) isolated from *met8-1* strain was not detectably suppressed by any of the antibiotics active on the parent strain (B).

We have tested several aminoglycoside antibiotics for translational misreading, as detected by incorporation of leucine directed by poly(U) which does not contain leucine codons. As shown in Fig. 2, hygromycin B, paromomycin and lividomycin B, all of which caused phenotypic suppression, stimulated misreading 2- to 3.5-fold. The same drugs caused misreading when added to a natural mRNA-stimulated system (L. Gritz, unpublished observation). Streptomycin, which does not suppress any of the nonsense mutations, had no effect on eukaryotic translation. The mechanism of misreading by aminoglycoside antibiotics is not known; the interactions between these drugs and pro- and eukaryotic ribosomes are presumably different, since different structure-function relationships are involved. The analysis of the ribosomes of resistant mutants should be

**Table 2** Aminoglycoside antibiotics tested for phenotypic suppression activity

| Antibiotic class                                   | Active                                                                                                                     | Not active                                                    |
|----------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------|
| The 4,5-disubstituted deoxystreptamine antibiotics | Neomycins B and C, lividomycins A and B, paromomycin                                                                       | Butirosins A and B                                            |
| The 4,6-disubstituted deoxystreptamine antibiotics | Kanamycins A and B, tobramycin, gentamicins X <sub>2</sub> , C <sub>1a</sub> , C <sub>2</sub> , A <sub>1</sub> ; sisomicin | Kanamycin C, gentamicins A, B C <sub>1</sub> , A <sub>2</sub> |
| The monosubstituted deoxystreptamine antibiotics   | Destomycin A, hygromycin B                                                                                                 |                                                               |
| Others                                             |                                                                                                                            | Fortimicin A, kasugamycin, streptomycin                       |

profitable in this respect. Hygromycin B has been shown to be an inhibitor of translocation in eukaryotic translation<sup>18</sup> and slowing of this process by the drug may increase the rate of incorporation of incorrect amino acids.

Our studies provide a basis for the genetic and biochemical analysis of mutants which may affect fidelity of translation in yeast and it should be possible to isolate and analyse mutants of the ribosomal ambiguity type in *S. cerevisiae* that have been exploited by Gorini and his coworkers in *E. coli*. In addition, antibiotic-induced phenotypic suppression may provide a convenient and quick screening method for nonsense mutants in eukaryotes. Paromomycin is a potent agent for phenotypic suppression and causes mistranslation *in vitro*, but has no measurable effect on *S. cerevisiae* growth. If the two processes are related, this eukaryote must be capable of tolerating extensive translational errors without apparent harm. This raises an interesting point with respect to the error catastrophe theory of translation errors in ageing.

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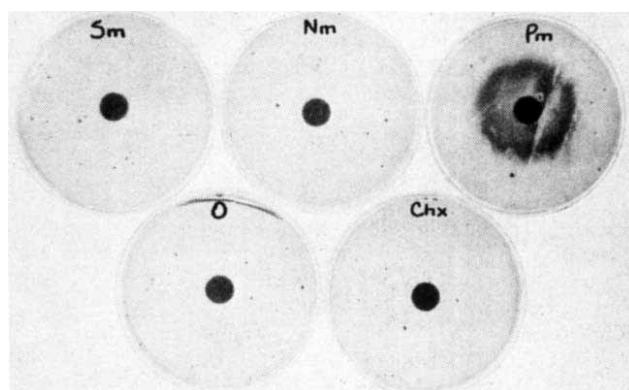
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## Phenotypic suppression of nonsense mutants in yeast by aminoglycoside antibiotics

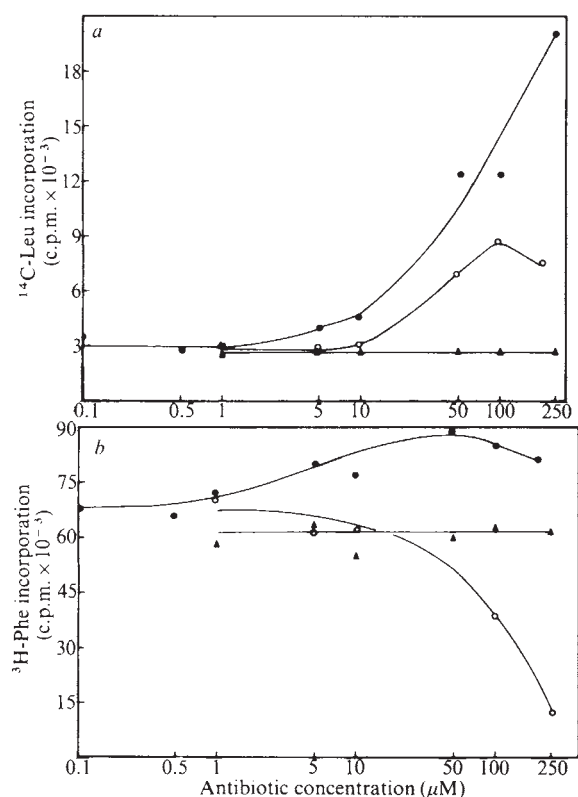
STREPTOMYCIN, an aminoglycoside antibiotic, can reverse the mutant phenotypes of many nonsense and missense mutations in *Escherichia coli* and in bacteriophage T4. This phenomenon has been called phenotypic suppression, since the mutant phenotype returns after removal of the drug<sup>1</sup>. The most likely explanation for phenotypic suppression is that streptomycin promotes mistranslation *in vivo*, and that acceptable amino acids are inserted into the growing polypeptide chain at the site of the mutant codon. Consistent with this view is the observation that streptomycin causes *E. coli* ribosomes to mistranslate RNA *in vitro*<sup>2,3</sup>. Streptomycin and neomycin have however been found to have no effect in stimulating ribosomes from eukaryotic cells to mistranslate RNA *in vitro*<sup>4,5</sup>. A subclass of the aminoglycoside antibiotics has been shown<sup>6,7</sup> to stimulate eukaryotic ribosomes to misread RNA. The highly active molecules are distinguished in that they contain the drug fragment paromamine (or 3'-deoxyparomamine). We have therefore examined the capacity of various aminoglycosides to suppress mutations phenotypically in the eukaryotic yeast, *Saccharomyces cerevisiae*. The results presented here show that paromomycin, which contains paromamine, is capable of phenotypic suppression of the nonsense mutations in *S. cerevisiae*.

The data of Fig. 1 show the relative ability of various antibiotics to suppress the methionine requirement of a yeast strain carrying the amber mutation *met8-1*. It is clear that in the presence of streptomycin, distilled water or cycloheximide, only rare isolated revertants appear, whereas paromomycin, one of the paromamine-containing drugs, supports a dense mat of growth around a paper disk containing this antibiotic. Although it is not obvious in the figure, neomycin causes a limited ring of growth around the disk.

Singly-cloned cells from the plate containing paromomycin were isolated and tested for their methionine requirement. All of the tested clones were still auxotrophic for methionine, indicating that paromomycin had not induced any permanent revertants. Additional nonsense mutants were tested for their ability to be phenotypically suppressed. The results (Table 1) indicate that 14 of the 17 mutants tested can be phenotypically suppressed with paromomycin, whereas only six mutations are suppressed with neomycin, albeit weakly. The mutations suppressed by paromomycin represent lesions in eight different metabolic pathways and in 11 different structural genes. It is therefore highly unlikely that paromomycin is directly supplying the growth requirements for all of the mutants. Paromomycin



**Fig. 1** Approximately  $10^7$  cells were plated on synthetic media<sup>13</sup> containing no methionine. A paper disk, saturated with  $7 \mu\text{mol}$  (5 mg except cycloheximide 2 mg) of antibiotic, was placed in the centre of each plate and the plates were incubated at  $30^\circ\text{C}$  for 3 d. Sm, Streptomycin; Nm, neomycin; Pm, paromomycin; Chx, cycloheximide; O, distilled  $\text{H}_2\text{O}$ .



**Fig. 2** Yeast cells (strain HL100-1C) were grown in standard nutrient medium (1% yeast extract, 2% peptone, 2% dextrose) to  $8 \times 10^7$  cells ml<sup>-1</sup>, collected and washed twice with distilled H<sub>2</sub>O. The cells were resuspended in homogenising buffer (20 mM HEPES, 4 mM Mg acetate and 100 mM KCl, pH 7.6) and disrupted with glass beads in a Braun mechanical cell homogeniser. The preparation of the extract and the conditions for cell-free protein synthesis are described elsewhere<sup>6</sup>. The final concentrations of Mg acetate and KCl in these yeast cell-free systems were 15 mM and 100 mM respectively. *a*, Poly(U)-dependent misincorporation of leucine; paromomycin (●); neomycin (○); streptomycin (▲). *b*, Poly(U)-dependent incorporation of phenylalanine; paromomycin (●); neomycin (○); streptomycin (▲).

can also suppress all three types of nonsense codons, UAG, UAA and UGA (Table 1). The strongest growth response to paromomycin was given by the markers *met8-1* and *trp5-48*, neither of which mutation could be suppressed by streptomycin (Fig. 1 and data not shown).

Growth in liquid cultures indicates that paromomycin can exert suppression at concentrations as low as 50 μM (35 μg ml<sup>-1</sup>). The concentration of paromomycin for optimal suppression of most mutations is approximately 100 μM.

Cell-free translation systems were prepared from yeast and the ability of paromomycin, neomycin and streptomycin to cause *in vitro* misreading was measured by the misincorporation of leucine with a poly(U) template (UU<sub>2</sub>pyr codes for phenylalanine, whereas CUN and UU<sub>2</sub>pyr code for leucine). The data in Fig. 2a show that paromomycin is active in stimulating leucine errors, yet streptomycin has no activity. Neomycin can stimulate leucine errors, although its activity is substantially weaker than that of paromomycin, especially at low concentrations. The data of Fig. 2b show that concentrations of neomycin which stimulate misreading also inhibit protein synthesis, whereas paromomycin stimulates mistranslation without inhibiting cell-free protein synthesis. This inhibition may account for the very limited phenotypic suppression seen with neomycin, even though the drug has some misreading capacity *in vitro*.

Phenotypic suppression can occur by several distinct mechanisms. The phenotypic reversal of mutant characters in yeast by hypertonic media is interpreted as an elevation of the osmotic pressure optima of certain mutationally altered proteins<sup>8</sup>. 5-Fluorouracil and related analogues are believed to

**Table 1** Phenotypic suppression of nonsense mutations in yeast by aminoglycoside antibiotics

| Marker and type | Growth supported by antibiotic<br>Paromomycin | Neomycin |
|-----------------|-----------------------------------------------|----------|
| <b>UAG</b>      |                                               |          |
| <i>met8-1</i>   | +                                             | trace    |
| <i>aro7-1</i>   | +                                             | trace*   |
| <i>ilv1-1</i>   | +                                             | 0        |
| <i>trp1-1</i>   | +                                             | 0        |
| <i>ade3-26</i>  | +                                             | 0        |
| <b>UAA</b>      |                                               |          |
| <i>lys1-1</i>   | +                                             | 0        |
| <i>trp5-48</i>  | +                                             | trace    |
| <i>ade2-1</i>   | 0                                             | ND†      |
| <i>his5-2</i>   | trace*                                        | 0        |
| <i>ura4-1</i>   | 0                                             | ND       |
| <i>lys2-1</i>   | +                                             | 0        |
| <i>ilv1-2</i>   | +                                             | 0        |
| <i>arg4-17</i>  | 0                                             | ND       |
| <i>leu2-1</i>   | +                                             | trace    |
| <b>UGA</b>      |                                               |          |
| <i>leu2-2</i>   | +                                             | trace*   |
| <i>lys2-101</i> | +                                             | trace    |
| <i>his4-166</i> | trace*                                        | 0        |

Nonsense mutations in *S. cerevisiae* which can be suppressed with paromomycin or neomycin. Cells were plated on the appropriate omission media for the indicated markers and incubated for 7 d with antibiotic containing disks as described for Fig. 1.

\* Phenotypic suppression of the indicated marker only seen in cells containing the extrachromosomal element  $\psi^+$  (in preparation).

† Not determined.

reverse mutant phenotypes in bacteria<sup>9,10</sup> and in *Neurospora crassa*<sup>11</sup> temporarily, by incorporation into mRNA, resulting in the pairing of 5-fluorouracil containing codons with incorrect tRNAs.

Since the paromamine-containing aminoglycosides bind to the cytoplasmic ribosomes of *Tetrahymena* and wheat embryos (unpublished observations), and since these drugs presumably bind to the cytoplasmic ribosomes of yeast, the suppression of many nonsense mutants by paromomycin reported here suggests that phenotypic suppression in eukaryotes can occur with agents which affect directly the functioning of the ribosome. It should be noted that Bayliss and Vinopal<sup>12</sup> reported the suppression of an *his1* missense mutant with streptomycin; however, extremely high concentrations of streptomycin were required to exert this suppression. It seems as if phenotypic suppression requires a 300-fold higher concentration of streptomycin, relative to paromomycin.

The paromamine-containing drugs should thus be useful in the isolation and characterisation of ribosomal mutants of eukaryotic cells which are altered in their translational fidelity. In addition, these agents should be helpful in the isolation of nonsense mutants and nonsense suppressors in eukaryotic organisms other than yeast. Most importantly, however, the paromamine-containing aminoglycosides should make possible a close examination of the consequences of protein synthesis errors in eukaryotic organisms.

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## X-ray absorption edge fine structure spectroscopy of the active site haem of cytochrome *c*

THE new technique of extended X-ray absorption fine structure spectroscopy (EXAFS)<sup>1</sup> offers promise of detecting small changes in conformation around a central metal atom at the active site of a metalloenzyme, and of relating such changes to the catalytic mechanism. An advantage of EXAFS studies over X-ray diffraction is that they can be carried out in solution at physiological pH where the protein can freely interact with its substrate. We describe here EXAFS measurements aimed at detecting conformational changes during reduction of horse heart cytochrome *c*.

Figure 1a shows the EXAFS-spectrum of reduced cytochrome *c* at pH 7, 20 °C, plotted against wave vector  $k = [(E - E_0)]^{1/2}$ .  $E$  and  $E_0$  are the X-ray photon and iron K-edge energies in eV respectively. X-ray photons with energies comparable to or larger than the Fe-1s-electron binding energy ( $E_0$ ) are absorbed by the iron thereby promoting the 1s electron to empty bound states (K-edge region) or into the continuum (EXAFS region). Two kinds of information can be deduced from an X-ray absorption spectrum. First, from the K-edge region information regarding the electronic state of the iron in the porphyrine frame and second, from the oscillatory region (EXAFS region) the mean bond lengths between the iron and its nearest neighbours (relative to a model compound used as standard). The technique has been reviewed in detail elsewhere<sup>1</sup>.

**Table 1**  $R_1 \approx R_1 - a/2$  for the first coordination shell [four pyrrole nitrogens, one nitrogen from His 18 and one sulphur from Met 80 (at low pH) or one nitrogen from Lys 79 (at high pH)]

|                                      |                             |
|--------------------------------------|-----------------------------|
| Reduced cytochrome <i>c</i> , pH 7*† | $1.47 \pm 0.03 \text{ \AA}$ |
| Oxidised cytochrome <i>c</i> , pH 7* | $1.47 \pm 0.03 \text{ \AA}$ |
| Oxidised cytochrome <i>c</i> , pH 10 | $1.40 \pm 0.03 \text{ \AA}$ |

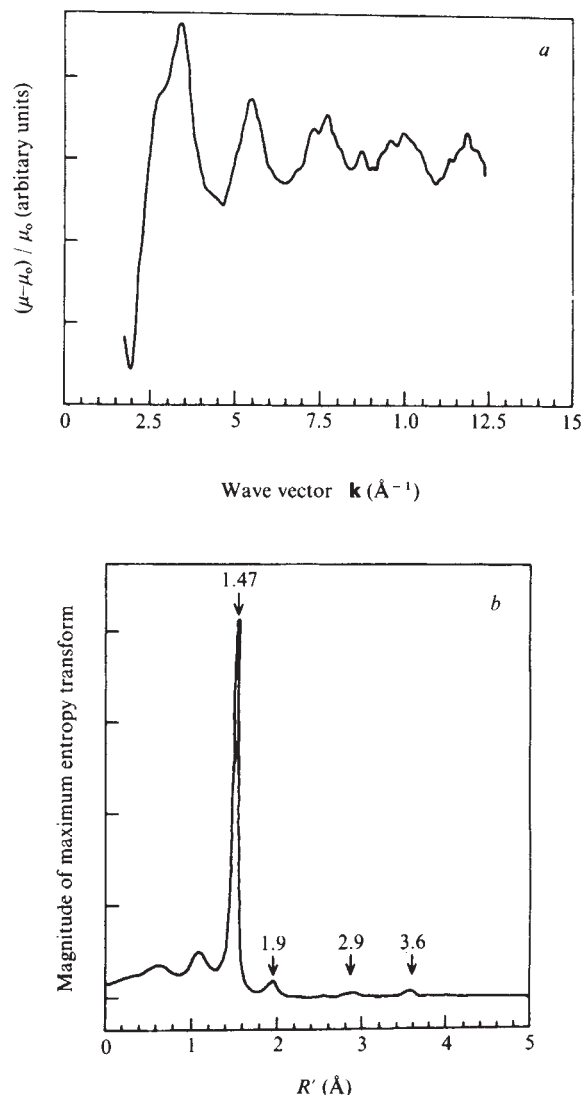
Values as obtained from maximum energy transforms<sup>7</sup>. The individuals atoms of the first coordination shell cannot be resolved.

\* In 0.1 M sodium phosphate buffer.

† Reduction was achieved by adding an equimolar amount of L ascorbate.

‡ In 0.1 M glycine buffer.

In native conditions (pH 7, 20 °C) the iron of cytochrome *c* is sixfold coordinated. Positions five and six are occupied by the  $\epsilon$ -nitrogen of His 18 and the sulphur of Met 80 (ref. 2). In this state it can be reversibly reduced and oxidised. The molecular details of this process are not fully understood. From antibody Fab fragment binding studies<sup>3</sup> it is known that human cytochrome has two different binding sites for the cytochrome *c* oxidase and the succinate cytochrome *c* reductase. The question has been raised as to whether there is a conformational change



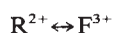
**Fig. 1** Iron K-edge fine structure spectrum of reduced horse heart cytochrome *c*, pH 7, 20 °C in 0.1 M sodium phosphate buffer. Concentration of cytochrome *c* is ~20 mM (a). The oscillation of the absorption cross section  $\mu$  is described by the semi-empirical formula

$$\frac{\mu - \mu_0}{\mu_0} = \sum_i \frac{N_i \cdot f(k, \pi)}{k \cdot R_i^2} \cdot D(k) \cdot \sin [2(R_i - a/2)k + b].$$

$\mu_0$  is the absorption in the absence of backscattering and  $k$  is the photoelectron wave vector.  $N_i$  is the number of backscattering atoms in the spherical shell of radius  $R_i$  surrounding the absorbing iron.  $f(k, \pi)$  and  $D(k)$  are the back-scattered amplitude and the Debye-Waller factor respectively. The maximum entropy transform<sup>7</sup> of the spectrum will as a function of the transformation parameter  $R'$  show peaks at  $R'_i = R_i - a/2$  (b). The phase shift  $a/2$  is given by the electronic inner shell properties of the absorbing and backscattering atoms and was therefore assumed to be the same for all samples measured. Changes in  $R_i$  are then equal to a corresponding decrease or increase in  $R'_i$ . The two small peaks in the transform at  $R' = 0.6 \text{ \AA}$  and  $1 \text{ \AA}$  are spurious and come from an improper background removal ( $\mu_0$ ).

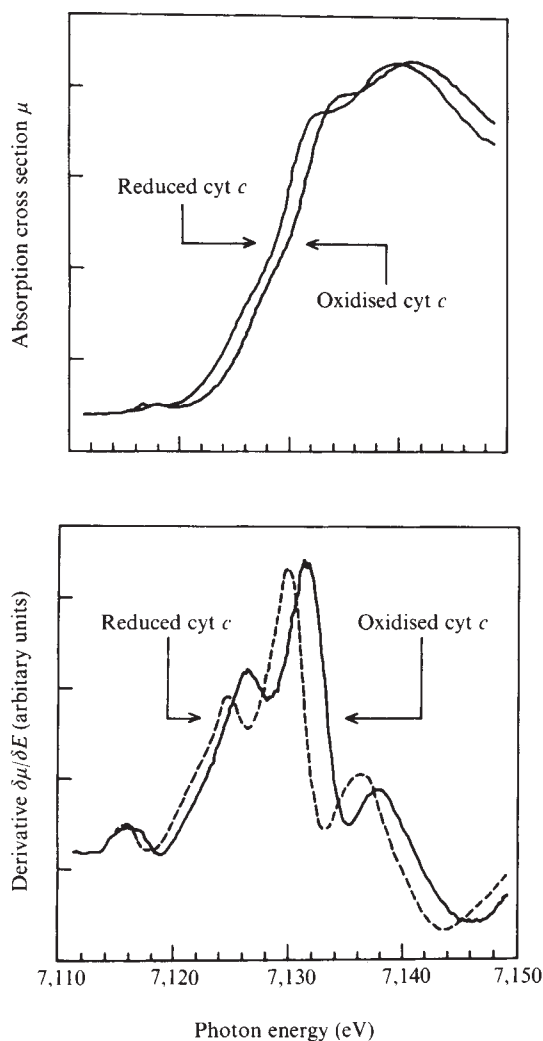
on reduction<sup>2</sup> which modulates the binding affinity of the two sites to their respective substrates. The latest X-ray diffraction results<sup>4</sup> on cytochrome *c* have a resolution of 2 Å and report the structures of ferric and ferrous cytochrome *c* from tuna which at this resolution do not differ significantly apart from the conformations of a few surface side chains. The early ferric horse heart map<sup>2</sup> showing considerable orientation changes in aromatic side chains and a slight main chain rearrangement to the first ferrous tuna map<sup>5</sup> is now believed to be incorrect.

The local resolution that can be obtained in EXAFS measurements is considerably higher than by X-ray diffraction. Any conformational change, occurring on reduction is expected to be triggered by the haem. Table 1 shows the uncorrected values  $R_1$  (see legend to Fig. 1 for the definition  $R'$  of  $R_1$ ) for the distance of the first coordination shell from the iron. These values were obtained by calculating the maximum entropy transform<sup>6,7</sup> of the EXAFS spectra as described previously<sup>8</sup>. The magnitude of the transform is plotted as a function of the transformation parameter  $R'$  in Fig. 1b. We estimate from their reproducibility that the  $R_1$  values are accurate to  $\pm 0.03$  Å. Within these limits we cannot see a conformational change in the haem region on reduction. On the other hand, H-exchange experiments<sup>9</sup> on horse heart cytochrome *c* show that the number of slowly exchanging amide protons is significantly higher for the reduced protein. This is interpreted by the authors<sup>9</sup> as unambiguous indication of a conformational rearrangement. In view of the current high resolution EXAFS results we would rather suggest a change in the amplitudes of some low frequency vibrations. The oxido-reduction then shifts the equilibrium between two conformers



(R standing for rigid and F for flexible) with almost the same time averaged coordinates. Such a 'conformational' transition can be brought about by a variation in bond density (rather than bond length) between the iron and its ligands. It is known that the haem plays an important part in stabilising the folding and that apo-cytochrome *c* does not fold at all<sup>10</sup>. Also the binding of

**Fig. 2** Iron K-edges and K-edge derivatives of reduced and oxidised cytochrome *c* and pH 7, 20 °C in 0.1 M sodium phosphate. Cytochrome *c* concentration is 20 mM.



**Table 2** Cytochrome *c* K-edge inflection point energies relative to the third inflection point of the K-edge of an iron foil (defined as 7130 eV)

|         | Oxidised<br>cytochrome <i>c</i> |         | Reduced<br>cytochrome <i>c</i> |         |
|---------|---------------------------------|---------|--------------------------------|---------|
|         | 1s-4s                           | 1s-4p   | 1s-4s                          | 1s-4p   |
| pH 1.5  | -2.4 eV                         | +1.5 eV | Unstable                       |         |
| pH 7.0  | -3.4 eV                         | +1.6 eV | -4.8 eV                        | +0.2 eV |
| pH 10.5 | -3.1 eV                         | +1.7 eV | -4.6 eV                        | +0.4 eV |

Buffers: 0.1 M glycine (low and high pH) and 0.1 M sodium phosphate. Cytochrome *c* concentrations range from 20 mM to 35 mM. No effect of the concentration on the K-edge positions could be detected.

*N*-acetyl-DL-methionine to the haem octapeptide (residues 1-8) has a smaller dissociation constant for reduced octapeptide<sup>11</sup>. Similarly in ferric cytochrome *c* the sulphur atom of Met 80 ligating the haem at neutral pH is replaced at high pH by a nitrogen (most likely from Lys 79)<sup>12</sup>. This exchange does not occur for ferrous cytochrome *c*. Table 1 gives the value of  $R'$  for this high pH ferric form of cytochrome *c*. The apparent shortening of the first coordination shell distance by 0.07 Å is marginal in view of the estimated uncertainty of  $\pm 0.03$  Å for  $R'$ . Whether the change in vibrational state or flexibility can alter physical properties such as the specific binding affinity has to await further elucidation.

Figure 2a shows the Fe-K-edge spectra of reduced and oxidised cytochrome *c* at pH 7. The edge structure is enhanced by differentiation with respect to photon energy (Fig. 2b). Three distinct transitions at photon energies smaller than the 1s electron ionisation energy can be seen. These transitions to empty bound states have been tentatively assigned to the 1s-3d, 1s-4s and 1s-4p transitions<sup>13</sup>. As seen from Fig. 2b there is a shift of the 1s-4s and 2s-4p transition by +2 eV on oxidation. This result is highly reproducible and in its most simple interpretation argues against extensive delocalisation of the electron added during reduction. It is in contradiction to results published earlier<sup>13</sup>, in which no change in electronic state was detected on oxidation. Moreover, we find practically no effect on the K-edge position when raising the pH, resulting in the exchange of the Met 80 sulphur by nitrogen. Table 2 gives the complete set of energies for the 1s-4s and 1s-4p transitions relative to the K-edge energy of an iron foil (7130 eV).

Note that we could not detect any degradation of cytochrome *c* after exposure to the high X-ray flux when checked by the optical absorption spectrum or the kinetics of ascorbate reduction<sup>14</sup>.

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# matters arising

## Was Venus born retrograde?

INGERSOLL AND DOBROVOLSKIS<sup>1</sup> have disputed my theory<sup>2</sup> of the atmosphere of Venus. The key quantity in both analyses is the accelerating atmospheric torque  $T_A$ , evaluated in equations (2) and (22) of refs 1 and 2 respectively. The latter expression is determined from observations to within some 30%, while the former contains the unknown 'thermal response time'  $\tau$  for mass transport in the atmosphere, for which the authors suggest values between some weeks and infinity. But sound waves can travel around the planet within some  $10^5$  s, hence I expect  $\tau$  to be about a day; and for this value, both determinations of  $T_A$  give the same answer.

The other important quantity is the braking solid body tidal torque  $T_B$  which depends on the average value of the quality factor  $Q$  divided by the tidal Love number  $k_2$ . Estimates for  $Q/4k_2$  range from 30 to  $\geq 10^5$  (refs 3, 2). In view of the different spin rate, chemistry and thermal history of Venus, a comparison with Earth need certainly not be trusted. An equality  $T_A \approx T_B$  can therefore at best be conjectured. In ref. 1, this equality is suggested to hold within less than 2%, an unlikely event without independent justification.

For this and the other reasons put forward in ref. 2, I still believe in the law of isochronism: that all Solar System secondaries were born with a prograde spin period between 5 and 10 h.

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INGERSOLL AND DOBROVOLSKIS  
REPLY—The questions which have been raised concerning the tidal torque on the atmosphere of Venus<sup>1</sup> deserve some clarification.

The atmospheric torque evaluated in equation (22) of ref. 2 depends on the coefficient of viscosity in the upper atmosphere of Venus. Because this is undoubtedly dominated by eddy momentum transport instead of molecular diffusion, the coupling between the upper

and lower atmosphere is uncertain by orders of magnitude, not by 30% as claimed by Kundt.

The thermal response time  $\tau$  is introduced in ref. 1 to parameterise unmodelled effects which tend to damp the atmospheric tides. It is not necessarily related to mass transport, and has nothing to do with the speed of sound waves (an entirely separate phenomenon). The main conclusions of ref. 1 (see our Fig. 2) are based on the undamped case  $\tau \rightarrow \infty$ .

Regarding the solid body torque, a value for  $Q$  of the order of 30 is not only suggested by analogy with the Earth, Moon and Mars, but is required (on the average) if Venus has despun from an originally rapid rotation, whether prograde or retrograde. Nevertheless, it is true that the near-equality (in the opposite sense) of the atmospheric and body tidal torque ( $T_A \approx T_B$ ) is a conjecture. This hypothesis was originally advanced by Gold and Soter<sup>3</sup> to explain the nonsynchronous rotation period of Venus.

These and similar questions are dealt with in greater depth in a forthcoming series of paper, as well as in ref. 4.

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## An alternative hypothesis for the origin of West African kimberlites

MARSH<sup>1</sup> suggested in 1973, that the alkaline intrusives along the coast of south-western Africa occurred where oceanic fracture zones extended under the adjacent continents. Recently Williams and Williams<sup>2</sup> have used this suggestion to attempt to explain the origin of the West African kimberlites. Their interpretation rests heavily on the accuracy of kimberlite and transform fault locations in their Fig. 1, on the assumption of Mesozoic ages for all of their kimberlites, and ignores the important occurrences in the same region of pre-Mesozoic kimberlites and alluvial diamond deposits.

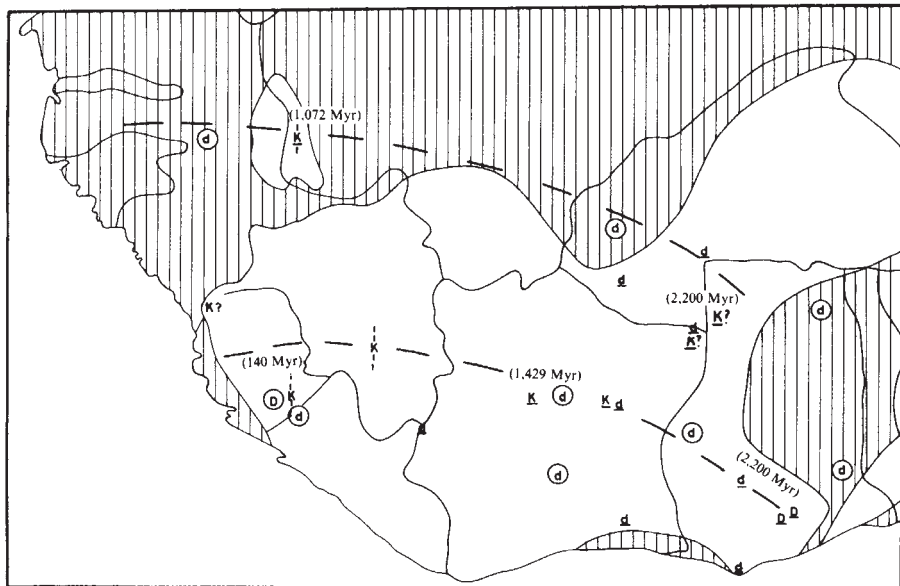
Williams and Williams' hypothesis assumes West African diamond deposits

to be Mesozoic, whereas the deposits in Ghana, Upper Volta, the Ivory Coast and Mali are Proterozoic or Infracambrian<sup>3–8</sup>. Their reference to kimberlites in Ghana is incorrect. Their location of a 'kimberlite' in Ghana fits the location of a few diamonds in a stream panning exercise<sup>3</sup> while the largest diamond fields of West Africa (placer), located in southern Ghana, are omitted. Their 'kimberlite' in Nigeria is a lamprophyre dyke located between Zaria and Sokoto (P. J. T. Verheijen, personal communication). Likewise, their postulated inland continuations of transform fault zones contradict without explanation the generally accepted trends<sup>9</sup> of these zones: the Chain and Charcot into the Benue trough,<sup>9</sup> the Romanche into the Keta basin bearing toward Ibadan,<sup>9,10</sup> and the St Paul entering the Ivory Coast east of Sassandra bearing ENE<sup>9,10</sup>. Thus the ages and locations of kimberlites as well as the positions of transform faults and their postulated landward continuations used by Williams and Williams<sup>2</sup> disagree with those established in the literature, with no explanation for this disagreement.

The geographical pattern of kimberlite pipes and the larger diamond placers can be more accurately interpreted (Fig. 1) as occurring along a primary arc running from Ghana through the Ivory Coast and Liberia to Sierra Leone<sup>11</sup>. In such a case the occurrence of young kimberlites is brought into coincidence with all of the much older kimberlites and alluvial deposits in the same general region. In addition there is a possibility of a second, roughly parallel arc extending from northern Ghana through Upper Volta, the Ivory Coast, Mali and Senegal. Known ages of major deposits along the primary arc include the diamonds contained in and above Birimian meta-sediments (2,200 Myr) in Ghana<sup>3,4</sup>, the Seguela and Kanangono kimberlites (1,429 Myr) in the Ivory Coast<sup>5,7</sup>, and the Sefadu and Koidu kimberlites (140–92 Myr) in Sierra Leone<sup>6</sup>. Along the second arc, the Kenieba kimberlites of Mali have been dated at 1,072 Myr (ref. 5). The geology of these deposits and the age determination techniques and results are discussed elsewhere<sup>3–8</sup>. Such a long history of progressive east to west arcuate diamond-bearing belts requires more than an association between Mesozoic kimberlites and post-Palaeozoic ocean fractures.

An equally viable explanation might be the existence of a long-lived mantle





**Fig. 1** Diamond and kimberlite occurrences in West Africa. K designates kimberlites (kimberlite belts are denoted with vertical caps and bases), D denotes major secondary diamond deposits, d marks minor secondary diamond occurrences. Underlined symbols are Precambrian in age, other deposits are younger. Circled symbols are alluvial deposits without age determinations; vertically shaded areas are covered with platform or basin sediments. Dates are given for diamond districts in which determinations have been made.

plume<sup>12</sup> producing a kimberlitic magma<sup>13-15</sup>. Then the alluvial deposits and kimberlites of the primary arc could be explained by the slow migration of such a plume from Ghana across the Ivory Coast to Sierra Leone with a long-term relative rate of motion of  $0.8 \text{ mm yr}^{-1}$ . The deposits on the secondary arc could be explained and would be consistent with a second but possibly weaker plume located north-west from the first plume, roughly in line with the Atlantic continental marginal intersections of the two arcs.

Such a hypothesis is consistent with the longevity, movement, and episodic nature of other hot spots such as the Hawaiian Chain<sup>16</sup>. The intrusion through the continents may be more episodic in nature, being shut off during periods of continental compression and opening up during periods of continental tension. Following the splitting of Africa from South America the rate of relative movement of the plume should have increased greatly. This would place the present position of the hypothetical plume, by extrapolation of the primary arc, at St Paul's Rocks on the Mid-Atlantic Ridge. This is known to be an active hot spot<sup>17</sup> and includes rocks with close affinities to kimberlites and carbonatites<sup>18</sup> as well as uplifted mantle material<sup>19</sup>.

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H. R. AND R. A. WILLIAMS REPLY—Hastings and Sharp<sup>1</sup> have attempted to explain the distribution of Precambrian to Mesozoic kimberlites in West Africa by means of two long-lived mantle plumes, the major one tracking from Ivory Coast to Sierra Leone with time. Their criticism of our hypothesis<sup>2</sup> that kimberlites occur along fundamental crustal fractures affected by oceanic lineaments, is unacceptable, especially as our hypothesis has been confirmed by Stracke<sup>3</sup> in south-east Australia, following his visit to Sierra Leone.

Hastings and Sharp have criticised our choice of data, particularly the ages of

kimberlites, their location, and that of transform fracture zones. With regard to the first, the reliability of much radiometric dating of kimberlites and other lithologies is fraught with problems<sup>4-7</sup> as shown by kimberlites in Mali, Liberia and Ivory Coast. Argument over the locations of kimberlites and fracture zones probably stems from the small-scale of our Fig. 1. However, some loci of our hypothetical continental continuations of transform fracture zones are ill-defined, but this is due to the scarcity of readily accessible, reliable Geological Survey data in West Africa. We did not include the diamond fields of southern Ghana in our Fig. 1 because we were discussing only the distribution of Mesozoic kimberlites. We are fascinated by the Nigerian kimberlite which has allegedly become a lamprophyre, but this does not subdue our hypothesis, as Scott<sup>8</sup> relates lamprophyres to kimberlites by a simple crystal fractionation process.

With regard to movement of mantle plumes over a period of nearly two billion years, we suspect that an arcuate locus is facile. The movement of the African plate since the Palaeozoic has been roughly northwards, as shown by the building of the Alps, the age succession of the Nigerian Younger Granites, and by palaeomagnetism. If Hastings and Sharp seriously consider that since the mid-Proterozoic plate movement relative to an assumed hot spot may be described as an arc, then we refer them to refs 9 and 10 for illumination.

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## The problem of thrown string

BASS AND BRACKEN<sup>1</sup> have proposed a probabilistic model to explain existing experimental results on the average length between the ends of a string when thrown onto a table. The theoretical values obtained from their model are not consistent with the available experimental evidence. However, the model presented seems to be a plausible description of the physical situation. We suggest here a

modification which takes into account the effect of the desire of the experimenter to obtain a random set of throws. We argue that the experimenter tries in his throws to obtain initial distances between the ends of the string (by initial we mean the distance just before the string begins to fall) which follow a uniform distribution. We contend that in this case the distribution of distances between the ends of the string just before it hits the ground is a mixture of equal proportions of a uniform distribution and the distribution proposed by Bass and Bracken<sup>1</sup>. If this argument is true the density function for  $\lambda$ , the ratio of the distance between the ends of the string at the end of each experiment to its total length, is

$$\omega(\lambda) = \frac{3}{4} \left[ \frac{5}{3} \arccos \lambda - \lambda(1 - \lambda^2)^{\frac{1}{2}} \right]$$

This gives  $E(\lambda) \approx 0.343$  and  $E(\lambda^2) \approx 0.178$  in striking agreement with the data. Bass and Bracken suggested that a proportion of the throws were conducted close to the table surface and if this were so, their model would give a better agreement with the data. It is easy to show that 38.5% of the throws are required to be near the table surface in order to give a good agreement with the experimental results.

We feel that our assumption is more realistic. However, the controversy on this problem will only be resolved when new experimental results are obtained and all the details of the experiment become available.

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**BASS AND BRACKEN REPLY**—Synge<sup>1</sup> has noted and rejected in passing the unfounded hypothesis that all possible end-to-end distances  $D$  of the string of length  $L$  lying on the table are equally probable (leading to  $\bar{D}/L = \frac{1}{2}$ ). A modified version of that hypothesis would be that all possible end-to-end distances of the string in space (before impact with the table) are also equally probable. Taking into account the projections of the string on to the plane table<sup>2</sup>, the probability density function for the ratio  $\lambda = \bar{D}/L$  is then  $\arccos \lambda$  (leading to  $\bar{D}/L = \pi/8 \approx 0.39$ ). The implausibility of the hypothesis leading to this distribution is apparent from the consideration that the number of configurations of the string consistent with an end-to-end distance  $x$  is much greater when  $x$  is near 0 than when  $x$  is near  $L$ .

If a fraction,  $p$ , of the throws conformed to our model<sup>2</sup>, and  $1 - p$  to the hypothesis of equiprobability of end-to-end distances

(in space), the resulting probability density of  $\lambda$  would be the combination

$$\frac{3p}{2} [\arccos \lambda - \lambda(1 - \lambda^2)^{\frac{1}{2}}] + (1 - p) \arccos \lambda;$$

and if  $p$  could be treated as an adjustable parameter, agreement with observations could obviously be improved<sup>3</sup>. We have not been able to find any rational basis for such a combination with  $p \neq 1$ . In particular, we cannot see that the choice  $p = \frac{1}{2}$  would arise from the circumstance that “the experimenter tries in his throws to obtain initial distances between the ends of the string ... which follow a uniform distribution”<sup>3</sup>.

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## Is lymphocytic chalone activity restricted to a spermine–protein complex?

THE nature of lymphocytic chalones which are supposed to be tissue-specific but not species-specific inhibitors of cell proliferation, as defined by Bullough<sup>1</sup>, remains a matter of controversy. They were characterised as 50–30,000-dalton factors<sup>2,3</sup> but detected also in the lower molecular weight range<sup>4,5</sup>. Recently Allen *et al.*<sup>6</sup> have proposed spermine as one candidate for an *in vitro* chalone activity. Spermine, present in a porcine thymus extracts as a protein complex, inhibited *in vitro* lymphocyte mitogenic response only when fetal calf serum (FCS) was added to the culture medium. FCS seems to act through polyamine oxidation, and its requirement for the inhibitory activity of spermine as well as spermidine was confirmed by Byrd *et al.*<sup>7</sup>. On the other hand, Rijke and Ballieux<sup>8</sup> have found a chalone-like non-dialysable factor in calf thymus extract differing from spermine complex in that its *in vitro* activity was FCS independent.

We have studied a non-dialysable fraction extracted from bovine spleen characterised previously as a lymphocytic chalone<sup>3</sup>. We demonstrated that most of the activity could be dissociated from high molecular weight carriers and recovered in a low molecular weight fraction<sup>5,9</sup> exhibiting chalone activity<sup>10</sup>. After chromatography, this fraction gave a purified spleen extract (PSE) whose effect was then compared with those of spermine and spermidine in mice. PSE ( $2 \times 25 \mu\text{g}$ ) was injected intraperitoneally. Twenty-four hours later, spleen, testis, kidney and liver cells were incubated with <sup>3</sup>H-thy-

midine. The radioactivity incorporated into DNA of cells was significantly reduced for spleen (39%,  $P < 0.005$ ) but not in liver, testis and kidney cells. PSE treatment depressed both the number of plaque-forming cells of sheep red blood cell-sensitised mice to 80% ( $P < 0.001$ ), as well as cellular immunity to dinitrofluorobenzene up to 51% ( $P < 0.01$ ), as measured by ear thickness in sensitised mice. Conversely, as judged from the same tests, no significant difference was observed between polyamine-treated mice. These results demonstrate that various factors different from spermine exhibit chalone activity.

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**ALLEN REPLIES**—I believe that inhibitors of lymphocyte mitosis are not restricted to spermine–protein complexes. In our article<sup>1</sup> we described how the activity of lymphocyte chalone preparations of the type originally used by Garcia-Giralt *et al.*<sup>2</sup> and Lenfant *et al.*<sup>3</sup> could be almost entirely attributed to the presence of spermine. We felt that ‘these results emphasise the difficulties associated with the isolation of a lymphocyte chalone, but by no means rule out its existence’<sup>1</sup>. Several groups, having heeded our warning and having now eliminated polyamines from their extracts, are finding that inhibitory activity persists. Dr Lenfant’s material is interesting in that it seems to be inhibitory both to the proliferation and function of lymphocytes.

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# reviews

## Development of preparative techniques

Savile Bradbury

*A History of Microtechnique.* By B. Bracegirdle. Pp. 359. (Heinemann: London, 1978.) £22.50.

STUDENTS of the history of biological science have devoted considerable attention to the microscope, both to the mechanical development of the stand and to consideration of the resolving power of the optics. Very little by comparison has been written about the equally important topic of the preparation of material for examination. This is an essential part of microscopy, just as much as interpretation of the image produced by the instrument. A knowledge of the development of preparative techniques throughout the period in which the microscope has been in use is, therefore, desirable. Such information, amply provided in this book, helps the historian decide to what extent the performance of the actual microscope was a limiting factor in the acquisition of data, whether there was a conceptual barrier in the mind of the observer or, as may prove likely, it was a combination of these allied to poor and limited preparative procedures. For example, some early techniques for preparing soft tissues such as striated muscle fibres involved their immersion directly in hot balsam resin. As Dr Bracegirdle vividly illustrates, such a preparation dated from 1869 shows little recognisable detail even when viewed by a modern instrument, whereas a microscope of 1826 gives a clear and detailed image of a modern stained preparation of striated muscle. This practical approach characterises the book, whose author examined over 40,000 slide preparations (some 3,500 in detail) during the course of its preparation; in addition he studied microtomes and other instruments in various collections in this country and abroad, and indeed succeeded in operating many of them in accordance with the instructions provided by their inventors.

Although there is clear evidence that microscopes were used to examine biological material from the middle of the seventeenth century onwards, few details of the preparative approaches used at that time survive. This may perhaps be attributed to the fact that very little in the way of specimen preparation was attempted, most observers being content to examine with incident light the surfaces of objects or to study fine dissections at low magnifications. The author considers the early efforts at tissue preparation

taking the early period as extending up to 1830. Only sporadic advances such as the invention of clearing agents, the mounting of thin sections of objects between talc covers in wood sliders and methods of micro-injection are to be found during this period. Obviously very few workers were taking microtechnique seriously and although the microtome, one of our most useful preparative tools in microtechnique, had been invented, only three examples (those of Custance, Hill and Adams) are recorded. This early period was, as the author comments, devoted to "specific advances of varying worth, not assimilated into an armoury of techniques generally in use"; some advances of great significance were however made, such as the introduction of the thin glass coverslip in 1789.

The century following the development of the achromatic microscope in 1826 was a time of great experimentation in microtechnique. Many and various reagents were tried, together with a host of embedding media. Some of these proved suitable—for example, paraffin wax and celloidin—and have survived into current use, whereas others—such as shellac, pulped paper, copal varnish and even tinfoil—have proved totally unusable and have disappeared from current practice.

Dr Bracegirdle devotes a long chapter to a discussion of the development of instrumentation in the nineteenth century. This chapter is largely an account of the microtome in its many and various phases and represents fascinating reading for a modern histologist.

The development of suitable chemicals and instruments for specimen preparation naturally led to the production of a large literature, much of it in German, during the nineteenth century. In his survey of

this aspect of the subject, the author makes a very interesting comparison between the attitudes in England and on the continent of Europe between 1830 and 1910. In this country it was clear that the gifted amateur reigned supreme in the use of the microscope, pursuing the ultimate in resolution. The perfection of mounts (usually of diatoms) which allowed this seemed to be the aim. On the continent, especially in Germany, the value of the microscope was appreciated for the help that it could provide in elucidating the structure of both normal and diseased tissues. This was reflected in the rapid development of microscopic histology in the medical schools on the continent and by the contributions of workers such as Purkinje, Henle, Kölliker and Virchow.

This is a fascinating book, not only for the historian of science but also for the general histologist. It uses the history of microtechnique, as it developed in the nineteenth century, to pose the question whether many of the workers were not in danger of developing sheer technique and procedure at the expense of careful observation and experiment. Such a question is still relevant today.

The book is well produced and contains both name and subject indices, together with citations of over 1,000 original books and articles. The illustrations are all by the author; many are of material from his own extensive collection and are of a high standard, although some may find the placing of all the plates together in two adjacent signatures irritating.

Here we have a work which is sure to remain the standard reference in this field for many years to come. □

*Savile Bradbury is Lecturer in Human Anatomy at the University of Oxford, UK.*

## Implications of artificial intelligence

*Computer Revolution in Philosophy: Philosophy, Science and Models of Mind.* By A. Sloman. Pp.304. (Harvester: Brighton, UK, 1978.) Hardback £12.50; paperback £5.50.

THIS is an untidy, un-pompous, exciting, iconoclastic and exasperating book. The author, a professional philosopher as well as a research worker in artificial intelligence, is scathing about much of the prevailing methodologies of science, especially psychology and the social

sciences generally, but his approach is perhaps best indicated by the following quotation in regard to philosophy:

"I am prepared to go so far as to say that within a few years, if there remain any philosophers who are not familiar with some of the main developments in artificial intelligence, it will be fair to accuse them of professional incompetence, and that to teach courses in philosophy of mind, epistemology, aesthetics, metaphysics, and other main areas of philosophy, without discussing the relevant aspects of artificial intelligence will be as irresponsible as giving a degree course in physics which includes no quantum theory."



I am not competent to pass judgment on this sweeping prophecy, but there is no doubt that much interesting and convincing evidence and arguments tending to support it are presented. And on the way, a host of intriguing ideas and speculations, the majority of them not followed up alas, are thrown up.

Here, only a few examples can be given. Few philosophers of mind, not to mention neuropsychologists and experimental psychologists, have yet taken on board the insight provided by the fact that the same cognitive computer programme can be run successfully on different computers with quite different basic components and architecture. In other words, the specifically mental part of the system is in an important sense quite independent of the physical part (which of course is not to say that it can operate without a physical part). How this comes about, the notions of "virtual machine", and of levels of representation of the activity, connected by translators, compilers and interpreters, are of significance here. Consider also the implications for the doctrine of reductionism in the philosophy of science. If biological processes are computational processes running on a physicochemical system, then it is not necessarily the case that the nature of the physical world determines them.

Another important notion for the psychology and philosophy of mind is that of systems and mechanisms which have the property of "mutual recursion", that is, the relationships between any parts of the system are not confined to being disjoint, or part-whole or overlapping, but A can be a part of B which is at the same time a part of A, infinite regresses being avoided by stopping conditions. A characteristically fascinating aside of Sloman's here is the suggestion that neglect of this aspect of the mind might have profound implications for educational practice, in that cumulative educational programmes may be misguided: teachers should not be surprised at pupils getting things wrong when they are in the midst of learning a collection of mutually recursive concepts.

An example of how tantalising the author can be is in a chapter which contains a most original and searching analysis of the relationships between inferences of the analogical type, such as those using diagrams, in contrast to the more usual "logical" types. In the middle he makes the very interesting suggestion, without following it up at all, that the essence of our notion of causality may be the following: we talk of causes when we believe there is a representation of the process (in the analogical or other form) which enables the effect to be *inferred* in the broader sense of "logic" he has been developing.

In the hope that the above has given some sense of the flavour of the book,

I shall now give a very brief summary of its contents. The first section deals with the aims of science and its relation to philosophy, putting the emphasis on the study of possibilities rather than the search for laws, and on conceptual analysis; it incidentally argues for a closer community of aims among philosophy, science and technology. The next section first attempts (in a very ambitious way) to sketch the structure of a mechanism of general intelligence. Three successive chapters then discuss intuition and analogical reasoning, learning about numbers and its bearing on the many and very diverse philosophical views on the nature of number, and perception as a computational process. It seems clear that the latter topic (in which Sloman's own research has been mainly concentrated) has been the one that has stimulated his thinking most. The final chapter discusses a large set of classical problems of philosophy in the light of our present understanding of artificial intelligence. These include universals, free will and determinism, mathematical knowledge, and aesthetic experience.

The main criticism one might make of the book, as has probably already occurred to the reader of this review,

is that it tries to cover too much, with the result that it spreads itself too thin over the large majority of the issues it raises, and gives the impression of making sweeping judgments without sufficient backing. Sloman also has a maddening habit of producing frequently lists (sometimes as long as fifty items) of questions for the reader, most of which are indeed interesting and pertinent ones, but of few of them does he say anything more. In addition, nearly every one of them would probably require at least a day's solid thinking on the reader's part to get any benefit from. Also, future editions of the book (which I am confident will be called for) would be improved by correction of the inordinate number of misspellings, removal of howlers like attributing the explanation of the equivalence of mass and energy to the theory of *general* relativity, or stating that superconductivity was explained before it was observed, and also improving the legibility of the legends of the diagrams.

**Bernard Meltzer**

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## Mathematical approach to vectors

*Vectors in Three-Dimensional Space.* By J. S. R. Chisholm. Pp.293. (Cambridge University Press: Cambridge and London, 1978.) Hardback £15; paperback £4.95.

FEW can appreciate a Beethoven sonata by merely reading the score. It needs to be played by a gifted interpreter. Ease and familiarity with mathematical ideas come quickly to only a few. Many of us need to develop mathematical skills, but such skills are precariously based if we have no appreciation of the underlying mathematical logic. The interpreter is needed here too and J. S. R. Chisholm has demonstrated yet again that he has this gift. His book with Morris *Mathematical Methods in Physics*—now sadly out of print—was a model of its kind: clear and readable but paying attention always to the rigorous basis of the techniques. This new volume is aimed more directly at the mathematics undergraduate but it should be one of the 'lecturer's textbooks' for anyone teaching vectors to scientists and engineers.

Too often a mathematician's approach to vectors begins with an assertion of the general algebra of vector spaces, leaving the student to find analogies, if he can, with the

world of three dimensional vector quantities in which he lives. This book maintains a healthy link with intuition. The axioms of vector space algebra are introduced from the start, but the three-dimensional geometrical interpretation of them takes up much of the text, with line-drawings to illustrate many of the arguments.

The relationship between vectors and vector-quantities is clearly explained. After establishing the axioms and discussing the products of vectors, there is a chapter in which geometrical examples are used to illustrate the relationship of vectors to matrices, tensors, the rotation group and general linear transformations. Vector calculus is introduced with a discussion of the geometry of curves and surfaces, going on to the differential geometry of curves, surface integrals and volume integrals.

The final chapter on vector analysis introduces the div grad and curl of scalar and vector fields in three dimensions, with Stokes's Theorem (a more rigorous proof is given as an appendix), the Laplace operator and related theorems.

Most subsections of each chapter have worked examples and up to seven or eight problems. Short answers to many of the problems are given at the back of the book.

**David J. Miller**

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## Biology of cancer

*Cancer: A Problem of Developmental Biology.* By G. B. Pierce, R. Shikes and L. M. Fink. Pp. 242. (Prentice-Hall: Englewood Cliffs, New Jersey, 1978.) £11.65.

READERS of that neglected masterpiece, *The Crock of Gold* (J. Stephens, Macmillan, 1928), will remember the philosopher's comment (to his wife) on the porridge — "Perfection is finality, finality is death. Nothing is perfect. There are lumps in it." Like the porridge, *Cancer: A Problem of Developmental Biology* has some indigestible lumps in it, but for all that it is a useful book for those for whom it is intended (graduate students). Don't be put off by the modest aims outlined (one hopes by the publisher) on the book cover:

"The unifying theme of this modern approach to the study of tumors is development and neoplasia, treating carcinogenesis as a model of differentiation rather than as a neoplastic entity alone. Coverage includes chapters devoted to the early development of neoplasms, tumors as caricatures of tissue renewal, structure and biochemistry of neoplasms, origin of neoplastic stem cells, carcinogenesis, genetics and neoplasia, controls, immunity and neoplasms, metastasis, and cancer therapy.

"An appendix concerning various types of tumours also is included. Among the features: Emphasizes the importance of cellular interaction in tumors, showing their cellular heterogeneity and control mechanisms; Presents an excellent introduction to the clinical phenomenology of cancer, placing more specialized aspects in a useful perspective; Organized into a logical, unified, and complete volume that includes the results of most recent research; Provides a complete bibliography of sources of more specialized information; Includes a chapter on therapy and some brief descriptions on the commonly studied tumors."

It does discuss many of these aspects but not in exhaustive detail, in its 199 pages of text. It does not compare in depth with Foulds' *Neoplastic Development* (Academic, 1969) which is still the best general introduction for the specialist to the biology of cancer. *Cancer: A Problem of Developmental Biology* is designed as an introduction to the field and in this I think that it achieves its purpose. For the student of biology with a general interest in cancer or a neophyte about to plunge into some highly specialised branch of cancer research, Pierce *et al.* will provide a useful reminder that there is more to cancer than molecules and also remind its readers that many of the findings of earlier workers are still relevant.

The faults of the book are superficial irritants rather than major defects, but nevertheless they are important, particularly in a book intended for students. The emphasis on the interests of the

authors is understandable and not excessive, but the style sometimes grates. There has to be a better way of saying "respond negatively in terms of growth rate", particularly if one wants students to learn conciseness of expression.

A more serious criticism is concerned with minor but important inaccuracies and omissions. It is easy for a reviewer to carp over trifles, particularly when the authors are trying to compress a mass of information into a small space, but it is important not to mislead. A few examples will illustrate the point. The given definition of malignant tumours states that the malignant cells "are intrinsically dangerous because they are rapidly growing and quickly cause cachexia or they invade and metastasize. The intrinsically dangerous cells of malignant tumours invariably destroy their host unless they are either extirpated or killed". Although this is often so, the variation in growth rate of tumours is well known as is the whole concept of latency in tumours and of carcinoma *in situ*, which are in fact discussed elsewhere. The appropriate qualifying clauses would clarify the situation—important for those using the index to look for a definition of malignancy.

On the experimental side the authors suggest that transformation *in vitro* should produce a spectrum of cell lines, some

tumour producing, some not—"an idea that has escaped attention", although they quote elsewhere a paper describing this very phenomenon. Sadly (as the paper is one of mine) it is given as a reference to work it does not describe.

In the general discussion on spontaneous transformation *in vitro* the authors fail to mention that this is predominantly a feature of some rodent cells and does not occur in species in which cells have been shown to have a finite lifespan as in Hayflick's experiments with human cells. In the section on genetics and neoplasia a statement is made that "recently, viral particles, similar to those of mouse mammary tumour virus, have been discovered in the milk of a high percentage of Parsi women" (Moore *et al.*, 1971), with the implication that these particles are true viruses and associated with a high incidence of breast cancer in these women. Many would no longer accept these findings. Other examples can be cited.

In spite of the criticisms this could be a useful book but it would be greatly improved by a more critical assessment of statements, definitions and references.

L. M. Franks

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## High performance liquid chromatography

*High Performance Liquid Chromatography.* Edited by J. H. Knox. Pp. 203. (Edinburgh University Press: Edinburgh, 1978.) £5.

THIS book is based on the material of a course on high performance liquid chromatography (HPLC) given in 1977 at the Wolfson Liquid Chromatography Unit, of which Professor Knox is the director. It represents a commendable attempt to put both the theory and practice of the subject on a firm physico-chemical foundation, which the student can use to attack his own problems. The main theory of liquid chromatography is compressed into the relatively short chapter 2, followed by successive chapters on the familiar subdivision of chromatographic methods according to retention mechanism, adsorption, liquid-liquid partition, and ion-exchange (including ion-pair) and exclusion chromatography. These topics are very fully treated in the space available, and each chapter includes a table of the appropriate column packing materials commercially available.

Unfortunately the theoretical treatment

of chapter 2 is rendered unnecessarily confusing by an unfamiliar notation in which  $V_m$ , the volume of eluent within the column, is taken to include both the liquid in the interstitial spaces between the packing particles and the liquid within the stationary phase pores. Although the pore volume cancels out in the definition of phase capacity ratio  $k'$  (equation 2.2, p5), it can only be valid as a mean value in the definition of linear flow velocity  $\mu$  (equation 2.3). It leads to a new definition of zone capacity ratio  $k''$  on p7, which is almost immediately abandoned on p8, leading to the more usual relationships of equations (2.12–2.17). The concept is again introduced in the treatment of the column resistance factor on pp9–10. This notation is all the more surprising, as the authors found it unnecessary in their excellent theoretical treatment in an earlier book.

Perhaps the most unfortunate result appears however in chapter 7 on exclusion chromatography, where an essentially simple concept is confused by the introduction of three definitions for the distribution ratio between the phases (p69). This chapter also illustrates the preoccupation of practitioners of HPLC with the exclusion chromatography of synthetic polymers, usually uncharged and soluble only in organic solvents. Although there is a brief reference to the



exclusion chromatography of biopolymers in chapter 7, the treatment is in some respects misleading. For example, in the case of the gel media used with biopolymers, there is an increased zone dispersion, which has a good theoretical foundation in the polydispersity of retention sites (that is pores) in the stationary phase. This problem has been extensively discussed by Ackers and others. The consequent poor resolution has led to the virtual abandonment of exclusion chromatography for the determination of the molecular weights of biopolymers by biochemists, who now favour electrophoresis in a continuous molecular sieving medium for this purpose. Also contrary to the impression given on p76, adsorption problems are still severe with many proteins on inorganic exclusion media. Polymer chemists seem to be indeed fortunate in the exclusion media available to them.

Chapters 8–10 give a very good account of the hardware of HPLC, columns, injection systems, pumps and detectors. Perhaps uniquely, this section also includes a discussion on the use of post-column reactor systems, which may be increasingly used in the future to increase both sensitivity and selectivity. Although the authors seem to be optimistic about

the future development of many of the presently less common detectors, I feel that the current escalation in the cost of HPLC equipment may put many of these more sophisticated detectors out of the reach of any but specialised laboratories. From this aspect I consider the comparison of analytical methods in Table 11.1 rather biased in favour of HPLC, especially as regards specificity and cost. For example, a good radio-immunoassay can be at least as specific as an HPLC estimation, whereas instrumental costs have been overtaken by inflation during the time taken for publication of the book.

Applications of HPLC, particularly to pharmaceutical and clinical analyses, and a description of the Wolfson departmental course experiments conclude the book. The all-important topic of column packing is excellently covered, although late in the book.

As a comprehensive, modern and methodical treatment of all aspects of HPLC, this book has few rivals, especially at its very modest price.

C. J. O. R. Morris

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## Molecular damage after radiation

*Radiation Effects: ESR and ENDOR Analysis.* By H. C. Box. Pp. 280. (Academic: New York and London, 1978.) \$24.50.

THIS book describes the molecular damage which occurs in carboxylic acids, amides, amino acids, peptides, proteins, pyrimidines, purines and nucleic acids, when these substances are bombarded with high energy radiation. In addition to the immediate effect of the radiation, the secondary processes are described and some overall mechanisms are followed through. Most of this information is derived from a knowledge of what radicals are formed, in what yield, how quickly they react, and with what. It is the unique contribution of electron spin resonance (ESR) supplemented by ENDOR (a special form of nuclear magnetic resonance which uses the ESR signal as a means of detection), which makes this study possible.

The first five chapters describe the characteristics of the ESR and ENDOR methods. In this part of the book, hyperfine structure and  $g$  values are treated with fair rigour and the Hückel and INDO methods of computing spin

densities are compared with each other and with experiment. The next five chapters are concerned with the radiation effects and mechanisms while the last chapter sets all this into perspective with current and projected work on: radiation modifiers which might be used in cancer treatment; conversion of solar energy into chemical energy; the relationship between radiation-induced carcinogenesis and DNA repair processes; and radiation damage in electron microscopy.

In general the book is well balanced between theory and experiment. It is well written and makes a fascinating account of our knowledge of the molecular events which follow irradiation of biological material. There are lots of well labelled chemical formulae and plenty of tables of data for reference purposes in the text and in appendices; these will make the book valuable for active workers in this field. The reviewer personally favours a slightly more experimental approach and would have preferred to see a few more actual spectra, particularly ENDOR spectra and more details of the assignments on these. Space could have been found at the expense of some of the mathematical detail in the early chapters which many readers more familiar with cell biochemistry might find difficult and unnecessary to follow.

J. F. Gibson

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# obituary

## Ivor Isaac

IVOR ISAAC, Professor of Botany in the University College of Swansea and Vice-Principal of the College from 1973 to 1977 died on 4 August 1978 at the age of 63.

He was a Swansea man born, bred and educated in the City. From Dynevor School he entered the University College of Swansea in 1934 and graduated with first class honours in botany. Isaac then trained as a teacher and taught for a short time afterwards but in 1940 he was awarded an A.R.C. Scholarship to work at Cambridge on plant pathology under the supervision of Professor F. T. Brooks. Two years later, Isaac moved to East Malling Research Station as a plant pathologist where he continued his studies for a Cambridge PhD on the plant wilt diseases caused by the fungus *Verticillium*: these studies determined Isaac's subsequent career.

After three years at East Malling, Isaac returned to school teaching at Cheltenham but in 1947 he was appointed Senior Lecturer in Biology at Acton Technical College which was later developed into Brunel University. While there, Isaac was responsible for the planning of the new Department of Biology at Brunel. However, in 1950 the opportunity came to return to Swansea first as lecturer in botany and subsequently as reader and professor in the University College. There Isaac played his full part in the development of a rapidly growing department and he pursued his researches on *Verticillium* wilt diseases so successfully that he soon became recognised internationally as one of the leading experts on this important pathogen.

Isaac's early work centred on clarification of the taxonomy of *Verticillium* isolates. At that time, there was considerable confusion because it was not clear whether the two most destructive pathogens in the genus viz. *V. albo-atrum* and *V. dahliae*, were forms of the same organism or were two distinct species. By using morphological, physiological and pathological criteria, Isaac established convincingly that these organisms are distinct species. A distinguished fellow worker wrote subsequently that this work brought order out of chaos.

Isaac continued, with the help of research students, to study many

different aspects of the vascular wilt diseases caused by *Verticillium* both in the United Kingdom and overseas. The studies were extended to a wide range of host plants often of great economic importance, such as lucerne and potato. The studies resulted in a continuing series of papers covering the ecology and survival of the fungus in the soil, the etiology of the disease, methods of control and the prevention of dissemination.

Isaac's reputation as a plant pathologist led to invitations to visit and advise in several countries including Italy, Malaya, India and the United States. Shortly before his death he was collaborating closely with the U.S. Department of Agriculture on an outbreak of *Verticillium* wilt on American lucerne crops. He served on several committees concerned with plant pathology and he played a leading part in the establishment of the first International Conference on *Verticillium*.

Isaac was no narrow academic. In his early years he was a very considerable soccer player and he captained both the Cambridge University team and the war-time combined Oxford and Cambridge XI. In later years in Swansea he was always deeply concerned with adult education and he frequently taught extra-mural courses himself. He was a man with many friends. His warm sympathetic personality was combined with high academic ability and clarity of thought; these qualities were much appreciated by colleagues and by students at all levels. He is survived by his wife Catherine, by three sons and a daughter.

J. M. Milton

## D. W. Kent-Jones

DR DOUGLAS WILLIAM KENT-JONES, O.B.E., F.R.I.C., died peacefully in Ealing on 31 August 1978, aged 87. He was one of the pioneers of the application of science to cereal technology and was recognised internationally as an authority on cereal chemistry. Indeed his book *Modern Cereal Chemistry* testifies to his immense knowledge of his subject.

In the 1914-18 war he served in the Royal Fusiliers, the Special Brigade of

the Royal Engineers and the Royal Flying Corps. It was during this time he claimed he learned his great capacity for getting on with others, and it was this capacity that spurred him throughout his long and active life to give generously of his time to many professional bodies. Thus he was honorary Treasurer of the Royal Institute of Chemistry (1946-53) and its President from 1955-57. He was honorary secretary of the food division of the Chemistry Section of the International Union of Pure and Applied Chemistry, Chairman of the Council of the British Industrial Biological Research Association (1966-70) and its President from 1970-72. In 1971 he was awarded the biannual Gold Medal of the Society of Chemical Industry and in 1974 he received the O.B.E. for his services to the food industry.

During the greater part of this time he was (until he reached the age of 75) the senior partner of the highly successful analytical and consulting chemists, D. W. Kent-Jones and A. J. Amos. After the 1914-18 war he had been chemist, and later director, of a firm in Dover associated with the milling and baking industries and in 1931 he formed the practice, specialising in cereals, first from laboratories in Dover, later in Ealing, and finally the spacious and well-equipped premises in Dudden Hill Lane, London.

He learned his baking by working in small bakeries after his days work, and in 1935 published *The Practice and Science of Breadmaking*. From 1926-45 he was the Chief Examiner of Breadmaking to the City and Guilds of London Institute. During 1930-39 he travelled and lectured extensively, becoming involved in Procea Products Ltd. when in the Antipodes and when the Company was formed in the UK he became a director, and finally Chairman, retiring in 1965. In 1938 he was awarded the Belgian Affront medal, and was made Professor *honoris causa* of the Institut des Industries de Fermentation. In 1947, at the request of the Bread Manufacturers of New South Wales, he advised on the setting up of the Bread Research Institute of Australia.

Wherever he went he was interested in measuring the qualities of flour and their relation to bread, cake and biscuit baking characteristics and he pub-

lished many papers on this subject. In 1949 he gave the Cantor Lectures of the Royal Society of Arts. He is well known for the development, with his colleague W. Martin, of the Flour Colour Grader, and the instrumental techniques now used in determining flour quality owe much to him, and his partner's enthusiasm for technological innovations.

Kay-Jay (as he was mainly known) and his wife had no children, but within the family he gave great affection and loyalty; so too did he to his many professional and close friends. He found great joy in the companionship of the Savage Club and after retirement, through being given honorary memberships of the Flour Milling and Baking Industries Research Association, the British Industrial Biological Research Association, the American Association of Cereal Chemists and the Bread Research Institute of Australia, as well as the honorary Fellowship of the Institute of Food Science and Technology.

*J. B. M. Coppock*

## D. M. Dring

DR D. M. DRING, Mycologist, Plant Pathologist and Quarantine Officer at the Royal Botanic Gardens, Kew, died suddenly after a brain haemorrhage on 26 July 1978 at the age of 46.

Born in Peterborough he went to Exeter University and obtained an external London B.Sc. (1953). From 1953 to 1956 he was an Andrew Simons Scholar at the same University where he worked on *Mycosphaerella* disease of cauliflower for which he was awarded a Ph.D. (1958).

This led to a wider interest in plant pathology and from 1956 to 1960 he was plant pathologist to the government of Ghana with an additional duty to act as Director of the Botanic Garden at Aburi. From 1960 to 1961 he was Agricultural Officer to the United Nations Food and Agricultural Organisation in West Africa working on Kaincope disease, spending much time in the Camerouns and Togo. During his travels he became interested in the larger tropical fungi, especially those bizarre and brilliantly coloured Gasteromycetes belonging to the Phallales.

On return to the United Kingdom he was awarded a Senior Research Fellowship to work for three years at the Royal Botanic Gardens, Kew, on the fungi he had collected in West Africa. Later in 1965, he joined the Scientific Civil Service as a Senior Scientific Officer and became a per-

manent member of the staff at Kew, where his unique knowledge of plant pathology combined with an interest in taxonomy of higher fungi were invaluable. Indeed his experience of tropical plant pathology ideally fitted him for the job of Officer-in-Charge of quarantine in a rapidly expanding unit, and in 1973 he was seconded briefly to the Government of Fiji to advise on the setting up of a quarantine station.

Donald Dring will be remembered for his publications on Gasteromycete taxonomy which established him as one of the world authorities on this difficult group of fungi. These contributions included floristic studies of the species occurring in tropical Africa and Israel. He also published, with Dr H. Kreisel, a monograph of the genus *Morganella*. However, his chief interest lay in the elucidation of the complex structure, inter-relationships and taxonomy of the phalloids, especially of the family Clathraceae. His magnum opus *Towards a natural re-arrangement of the family Clathraceae* was in an advanced stage of preparation at the time of his death and it is hoped to publish this in due course.

Another of his many scientific activities involved giving a stimulating annual course of lectures on mycology to the student gardeners at Kew. He also took a keen interest in the affairs of the British Mycological Society and served as a co-editor of their Transactions from 1978–1971, and for part of that time as senior editor. His editorial expertise also led to his serving on the editorial board of the *Kew Bulletin*. He also served on the Council of the Systematics Association.

Donald Dring was a congenial colleague who was always happy to take part in and contribute to social occasions. He had a great interest in antiques and enjoyed restoration of period furniture. He leaves a widow, Vivienne, and two daughters.

*Derek A. Reid*

## A. S. Jack

ANTHONY JACK, member of staff of the MRC Laboratory of Molecular Biology, Cambridge, died of a heart attack on 14 July 1978 at the age of 30.

Born in Newcastle, he was educated at Leeds Grammar School and at Peterhouse, Cambridge, which he entered as an Exhibitioner in 1966. After getting a First in Part II of the Natural Sciences Tripos (Crystallography), he went on to the MRC Laboratory of Molecular Biology to work for a PhD which he received in 1972. He spent two years on a Jane Coffin Childs Re-

search Fellowship at the Gibbs Laboratory, Harvard University, and returned to the MRC Laboratory in 1974 as a junior member of staff.

Tony Jack's research work centred on the structures of large biological molecules and methods of solving them by X-ray analysis. Although a skilful experimenter when the occasion demanded, he was, by temperament and aptitude, more of the analytical type of macromolecular crystallographer, more intrigued by the way to the solution than by the results of the solution itself. Thus it was no coincidence that he was attracted to problems which were non-orthodox at the time. His PhD thesis, following work initiated by Blow, Rossmann and Crowther, deals with the problem of how to use for phase determination the redundant information present in the X-ray intensities of structures made up of many subunits. He solved a practical problem, namely a two-dimensional projection of the structure of the protein disk of tobacco mosaic virus, thereby pointing the way to the full solution of three-dimensional virus structures.

In the laboratory of S. C. Harrison at Harvard, he played a key role in the earlier stages of the X-ray work on tomato bushy stunt virus, among other things, using the low resolution structure reconstructed from electron micrographs to locate the heavy atoms used in the isomorphous replacement analysis.

On his return to Cambridge, Jack turned to the problem of refining the structure of the monoclinic form of tRNA, solved there earlier. This analysis, as well as settling the details of the tertiary interactions, led to the discovery of certain new correlations in nucleotide conformation and showed up the magnesium and spermine binding positions in the molecule. In an extension of this work on tRNA, Jack, together with M. Levitt, developed a particularly rapid and powerful method of refinement, which combines X-ray results and energy analysis. The method has been applied successfully to a number of proteins as well, and bids to become widely accepted.

Almost none of his friends or colleagues knew of Tony Jack's heart condition. Though quiet by nature, he was ever energetic and worked with astonishing rapidity. He was a man of varied interests. As an undergraduate he played in the 'Pineapple Truck' pop group, having built his own bass guitar. He was widely read in 18th century English literature, he collected its authors and was a stout Johnsonian. He is survived by his wife, Sharon Bellard, a chemical crystallographer, whom he met at Harvard.

*A. Klug*